H124/3820

No Restriction.

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON.................... 14 September 2004.................

.... ▮▮▮▮ ....
<u>Sec. Research Graduate School Committee</u>

RESPONSE TO EXAMINER'S SUGGESTIONS

1. The evaluation of the effects of PPAR-α ligands such as fibrates in atherosclerosis susceptible rodent models is complicated by the multiple and sometimes different metabolic effects in animals compared to humans. For example, ApoE$^{-/-}$ mice show an unusual increase in lipids (cholesterol and triglycerides) in response to fenofibrate treatment (Duez H et al (2002) J Biol Chem, 277:48051-48057). Despite the alterations in lipids in the opposite direction to that observed in humans, the mice show a decrease in cholesterol extracted from aortic vessels and a reduction in plaque area (Duez H et al (2002)). Thus, fenofibrate appears to have lipid independent vascular actions and these were the subject of the detailed biochemical studies in this thesis. Whilst the in vitro studies in this thesis have the advantage of being on tissue from a human source, they also provide mechanistic evidence to support the design of an appropriate study to look at the proteoglycan-lipoprotein retention in vivo which would strengthen our current knowledge and this is being planned for the near future. Assuming that the outcomes of the FIELD Study are positive and follow similar trends observed in the DAIS, the in vitro studies in this thesis will serve as a platform for understanding the vascular effects of fenofibrate and how it may reduce atherosclerosis in susceptible patients.

2. The Western blot of PPAR-α protein from human vascular SMCs (Figure 3-3, page 103) was not as high quality as we would have wished and does not permit for easy interpretation but was the best that could be achieved with the products available at the time. Nonetheless, this data serves as a supplement to existing knowledge that human vascular SMCs express PPAR-α (Staels, 1998). The observation that PPAR-α is expressed by human SMCs used for the studies is an adjunct to the mechanism by which PPAR-α ligands modify vascular proteoglycans but may not necessarily be the actual effect.

3. The analysis of proteoglycan core proteins was performed simultaneously with total protein analysis (refer to page 104, paragraph 1) and therefore does not allow for the analysis of cell number. Generally cell number can be used as a substitute for total protein synthesis hence, the variability in cell number if any is reflected in the measurement of total protein. The data

show that fenofibrate treatment did not significantly alter total cellular protein unless the concentration exceeded 50 µmol/L. No experiments were conducted with concentrations that caused excessive and undesirable changes in cell number.

4. Glycosaminoglycan release from core proteins using alkaline borohydride was characterized by Don M. Carlson in 1968 (J Biol Chem. 243: 616-626). While there may be some loss of sulfate groups during long incubations with sodium borohydride, the length of the GAG chains is not altered. Hence, the small changes in GAG length observed following the treatment of vascular SMCs with fenofibrate are not confounded by any degradation during the β-elimination reaction. In addition, the changes in GAG length are supported by other findings which show that SMCs treated with fenofibrate show no change in proteoglycan core protein size (Figure 3-8, page 114) and small changes in GAG length are observed with xyloside-initiated GAGs which are synthesized independently of the core protein (Figure 3-19, Page 136 and Table 3-1, Page 143).

5. The examiner is correct in pointing out that the size exclusion data does not allow for estimation to the level of three decimal points and the presentation of the data has been recorded more conservatively.

6. The duration of each enzyme reaction was 16 h which is in excess of the time suggested by the supplier of the enzymes (30 to 60 min). Excess incubation time with the enzymes was performed to ensure that the GAGs were completely and sufficiently digested for valid analyses.

7. In Part 5.2.2, the culture medium from monkey SMCs metabolically labelled with both [^{35}S]-sulfate and [^{3}H]-glucosamine was applied to a DEAE-Sephacel column and eluted with a sodium chloride gradient (0-0.7 M; page 228, Figure 5-3 and page 229, Figure 5-4). It has been previously shown using a mixture of glycosaminoglycans from monkey aorta and separation by NaCl gradient, that the peak which elutes at 0.1-0.3 M NaCl is hyaluronan using an absorbance assay for uronic acid (Radhakrishnamurthy *et al* (1977) Anal Biochem 82:445-454) and the peak which elutes at 0.25-0.6 M (peak 3) has been fully characterized to contain heparan sulfate, chondroitin sulfate/dermatan sulfate proteoglycans by Chang and colleagues (J Biol Chem (1983) 277:48051-48057).

8. TGF-β1 stimulation of vascular proteoglycan synthesis showed a maximum response at ~0.3-0.8 ng/mL, however in the experiments presented in other chapters, concentrations of 1 or 2 ng/mL were used. It has been previously shown that TGF-β1 concentration-relationships for promoting proteoglycan synthesis do not follow classical responses (Scott *et al.*, 1997) in that the higher concentrations inhibit proteoglycan synthesis. Our laboratory has shown that this occurs in the human SMCs used to study proteoglycans and more recent TGF-β1 concentration-relationships from our laboratory have shown that there is half a log unit shift in the peak concentration for proteoglycan stimulation compared to those presented in these studies. Nevertheless, the concentrations of TGF-β1 used in these studies always demonstrated increased proteoglycan synthesis above basal levels which was the aim of each experimental protocol.

This thesis is dedicated to my parents, Pasquale & Marcella Nigro
and to my soul mate, Brendan Brown who have shown their
unconditional love, support, and encouragement and taught me to never let it beat me.

The role of PPAR-α ligands (fibrates) in the regulation of vascular smooth muscle proteoglycan synthesis and structure as a contributor to reduced lipoprotein binding and the development of atherosclerosis

Julie Nigro

BAppSci (Med Lab Sci), BSc (Hons)

Department of Medicine (Alfred Hospital), Monash University

May 2004

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Chapter 7:   *Discussion*

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List of Abbreviations

AII	Angiotensin II
AMAC	2-aminoacridone
CABG	Coronary artery bypass grafting
CCAs	Calcium channel antagonists
CHD	Coronary heart disease
CPC	Cetylpyridinium chloride
CS	Chondroitin sulfate
DAIS	Diabetes Atherosclerosis Intervention Study
DMSO	Dimethylsulfoxide
DS	Dermatan sulfate
FACE	Fluorophore assisted carbohydrate electrophoresis
FFAs	Free fatty acids
FFB	Fenofibrate
FIELD	Fenofibrate and event lowering in diabetes
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GlcNAc	N-Acetylglucosamine
GMSA	Gel mobility shift assay
HA	Hyaluronan
HS	Heparan sulfate
IdoA	Iduronic acid
IMA	Internal mammary artery
KI	Protein tyrosine kinase inhibitor
KS	Keratan sulfate
LDL	Low density lipoprotein
PDGF	Platelet derived growth factor
PGs	Proteoglycans
PPAR	Peroxisome proliferating-activated receptor
SMC	Smooth muscle cell
TGF-β1	Transforming growth factor-β1

Summary

Fenofibrate, a PPAR-α ligand, is indicated for the treatment of hypertriglyceridemia in patients with diabetes. In addition to beneficial effects on lipids, fenofibrate and a similar agent gemfibrozil, have been shown to reduce macrovascular disease in clinical trials. Modified extracellular matrix secretion by atherogenic growth factors such as transforming growth factor (TGF)-β1 predisposing to increased lipoprotein retention has been proposed as a key event in the atherogenic process. We investigated whether or not fenofibrate has direct vascular actions on vascular smooth muscle cells (VSMCs) and specifically looked for effects on proteoglycan synthesis, structure and binding to low-density lipoprotein (LDL).

Human VSMC cultures were treated with fenofibrate (0.3-50 μmol/L) in the presence or absence of atherogenic growth factors, TGF-β1 and platelet derived growth factor (PDGF). Metabolic labelling with [^{35}S]-methionine/cysteine assessed core protein, [^{35}S]-sulfate or [^{3}H]-glucosamine assessed glycosaminoglycans (GAGs). Proteoglycans were assessed by the cetylpyridinium chloride (CPC) precipitation assay, SDS-PAGE and molecular sieve. The sulfation pattern of GAGs was assessed by Fluorophore Assisted Carbohydrate Electrophoresis (FACE) and GAG composition by enzyme digestion and SDS-PAGE. Gel Mobility Shift Assay (GMSA) assessed proteoglycan binding to LDL.

Fenofibrate (30 μmol/L) treatment of VSMCs increased core protein synthesis by 15.7% ($P<0.05$). Fenofibrate (50 μmol/L) treatment of VSMCs in the presence of TGF-β1, inhibited the incorporation of [^{35}S]-sulfate into secreted proteoglycans by 28.6% ($P<0.01$). Fenofibrate (50 μmol/L) treatment of VSMCs in the presence of PDGF, inhibited the incorporation of [^{35}S]-sulfate into secreted proteoglycans by 51.8% ($P<0.001$). The changes in sulfate incorporation following treatment with fenofibrate were associated with a

concentration-related increase in the electrophoretic mobility by SDS-PAGE due to a reduction in GAG length; there was no change in the sulfation pattern however, there was an alteration in the disaccharide composition of the GAGs. Proteoglycans synthesized in the presence of fenofibrate showed an increase in the half-maximal saturation concentration of LDL from 36.8 ± 12.4 μg/mL to 77.7 ± 17 μg/mL under basal conditions, 24.9 ± 4.6 μg/mL to 39.1 ± 6.1 μg/mL in the presence of TGF-β1 and 9.5 ± 4.4 μg/mL to 31.1 ± 3.4 μg/mL in the presence of PDGF/insulin.

Treatment of VSMCs with fenofibrate (50 μmol/L) increased [^{3}H]-glucosamine incorporation into GAGs by 95.6% ($P<0.05$). We investigated whether or not the specific activity of radiolabelled glucosamine is altered by treatment of vascular SMCs with fenofibrate. Proteoglycans were assessed with [^{35}S]-sulfate and [^{3}H]-glucosamine metabolic precursors and the specific activity of [^{3}H] was assessed by measuring the incorporation of [^{3}H] and [^{35}S] in fluorescently labelled derivatized (Δ) disaccharides, Δdi-6-sulfate (Δdi6S) and Δdi-4-sulfate (Δdi4S), using FACE. Fenofibrate treatment increased the specific activity of [^{3}H]-galactosamine in Δdi4S compared to the low glucose control from 48.2 ± 3.2 μCi/μmol to 57.1 ± 2.6 μCi/μmol ($P<0.05$). Treatment of VSMCs with fenofibrate showed increased glucose consumption under low (5.5 mmol/L) glucose conditions.

We conclude that fenofibrate alters glucose metabolism by human VSMCs which increases the specific activity of glucosamine used for the synthesis of GAGs. Fenofibrate modifies the structure of vascular proteoglycans by reducing the length of the GAG chains and GAG composition resulting in reduced binding to human LDL, a mechanism which may lead to a reduction of atherosclerosis and cardiovascular disease in high risk subjects treated with fenofibrate.

Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution. I affirm to the best of my knowledge that this thesis contains no material previously published or written by another person except where due reference is made in the text of this thesis. This declaration is the result of scientific research solely conducted by the author.
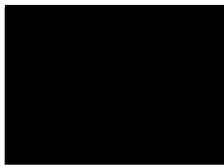
Julie Nigro

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Acknowledgment of assistance with specific work presented in this thesis

- Dr. Rodney J. Dilley assisted with the immunocytochemical staining of human vascular smooth muscle cells (Chapter 3, Figure 3-1).

- The resources of the Cell Biology of Diabetes Laboratory including the image of confluent serum deprived human smooth muscle cells (Chapter 3, Figure 3-2).

- In Chapter 4, Section 4.2.1, Paragraph 1 is summarised from our paper, Ballinger *et al*. The corresponding figures, Figure 4-1, Figure 4-2 and part of Figure 4-8 and Figure 4-10 have been previously published in the same paper.

- Ms. Stephanie deDios provided a gel image of proteoglycans from human smooth muscle cells treated with troglitazone (Chapter 5, Figure 5-1A) and also provided technical assistance with size exclusion chromatography and gel mobility shift assay

- The experiments conducted under Section 5.2.2, were performed in the laboratory of Professor Tom Wight under the supervision of Dr Susan Potter-Perigo, Hope Heart Institute, Seattle, USA. The assistance of Dr. Michael Kinsella, and Dr. Stephen Evanko, with the design of experiments and interpretation of results is acknowledged. Dr. Sana Sakr and Dr. Tom Wilkinson are acknowledged for technical assistance for the analysis of hyaluronan.

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- Ms. Melanie Ivey performed the glucose and lactate measurements presented in Chapter 5, Figure 5-10.

- Ms. Mandy Ballinger provided a gel image of proteoglycans from human smooth muscle cells treated with a kinase inhibitor (Chapter 6, Figure 6-11A) and is acknowledged for technical assistance with Western blotting and valuable discussions with the interpretation of results.

- The proteomics experiments conducted under Section 6.2.2.4, were performed in the laboratory of A/Prof. Ian Smith under the supervision of Ms. Shane Reeve. Ms Shane Reeve is acknowledged for technical assistance with 2-dimensional electrophoresis and performing the mass spectrometry (MS) analysis.

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- Dr. Geoff Head, Baker Heart Research Institute, established the spreadsheet for the statistical analysis using a 1Way ANOVA, presented in Appendix I.

Manuscripts relating to this thesis

Nigro, J., Dilley, R. J. and Little, P. J. Differential effects of gemfibrozil on human vascular smooth muscle cell migration, proliferation and proteoglycan biosynthesis. **Atherosclerosis (2002) 162: 119-129**

Little, P. J., Allen, T. J., Hashimura, K., **Nigro, J.,** Farrelly, C. A. and Dilley, R. J. High glucose and transforming growth factor-β1 independently potentiate the proliferation of ovine coronary smooth muscle cells. **Diabetes Research and Clinical Practice (2003) 53: 93-101**

Ballinger, M.L., **Nigro J.** Frontanilla, K. V., Dart, A. M. and Little, P.J. Regulation of glycosaminoglycan structure and atherogenesis. **Cellular and Molecular Life Sciences (2004)** (in press)

Nigro, J., Ballinger, M.L., Dilley R.J., Jennings G. L. R., Wight T.N. and Little P.J. Fenofibrate modifies human vascular smooth muscle proteoglycans and reduces LDL binding. **Diabetologia** (Submitted April 2004)

Nigro, J., Susan Potter-Perigo, Melanie E. Ivey, Stephanie T. de Dios, Garry L. Jennings, Stephen P. Evanko, Michael G. Kinsella, Thomas N. Wight and Peter J. Little. PPAR ligands modulate glucose and glucosamine metabolism and alter proteoglycan biosynthesis in human vascular smooth muscle cells. (In preparation)

Chapter 1: Literature Review

1.1   Diabetes and Obesity

1.1.1   Prevalence and Aetiology

Diabetes is a clinical condition where blood glucose levels are abnormally high and toxic to tissues. There are two forms of diabetes, type 1 and type 2 which both result in hyperglycemia. In Australia, 7.4% of the adult population ($\approx$1.2 million people) have diabetes, 15% of which have type 1 and the remainder have type 2 (Bate & Jerums, 2003; Dunstan *et al.*, 2002b). Type 1 diabetes is caused by pancreatic failure to synthesize insulin, the hormone that promotes glucose storage. In type 2 diabetes, the cells of glucose storing tissues, such as skeletal muscle and the liver, become insensitive to insulin and the pancreas initially compensates for the insulin resistance by increasing insulin production. Type 1 diabetes commonly occurs in children and young adults with a prevalence of 192 per 100,000 children, aged less than 20 years being affected (Cooper & Stroehla, 2003). Type 1 diabetes is increasing at rate of $\approx$3% per annum in young children worldwide (EURODIAB ACE Study Group, 2000; Green & Patterson, 2001; Onkamo *et al.*, 1999). Conversely, type 2 diabetes is most commonly diagnosed in patients above the age of forty years however, over the last 10 years increasing numbers of young children have been diagnosed with type 2 diabetes (Cruz & Goran, 2004; Cruz *et al.*, 2004; Gorman, 2003). The increasing rate of type 2 diabetes has coincided with detrimental environmental factors including sedentary lifestyle and poor nutrition which has also increased the rate of obesity, a condition known to exacerbate the development of diabetes.

There have been multiple hypotheses presented regarding the aetiology of both type 1 and type 2 diabetes however, a single cause for either disease has not been identified. For type 1 diabetes it is generally regarded that genetic predisposition is an important factor, in which 35% have a familial history of the disease (Vardaxis, 1994). Additionally, environmental

factors, such as an enterovirus, may initiate an autoimmune response that destroys the insulin producing cells (β cells) of the pancreas. For type 2 diabetes, it is mainly environmental factors such as sedentary lifestyles and obesity on a background of genetic susceptibility, which contribute to the development of the disease.

1.1.2 Signs and symptoms

During the early stages of type 2 diabetes, patients may be asymptomatic and may only be diagnosed following a clinical event, such as myocardial infarction. In untreated type 2 diabetes, the signs and symptoms have a slow onset but generally include polydipsia, polyuria, glycosuria, ketonuria, persistent skin infections, hypertension and vascular disease (Kaplan & Pesce, 1996; Vardaxis, 1994). Patients with type 2 diabetes are diagnosed through the demonstration of a fasting blood glucose level greater than 5.5 mmol/L. Diabetes is also diagnosed following an oral glucose tolerance test (OGTT) in which 50-75 g of glucose is given orally and peak blood glucose levels are greater than 8.8 mmol/L with a slow decline compared to normal subjects (Figure 1-1) (Kaplan & Pesce, 1996; Vardaxis, 1994). Following diagnosis, haemoglobin (Hb)A$_{1c}$ is used as an additional biochemical marker of blood glucose control with a target value of <7% (Mayer & Freedman, 1983).

Diabetes is also significantly associated with other risk factors such as high blood pressure, obesity and dyslipidemia (Adler, 2002) and this combination, with hyperglycemia, has been called the "Deadly Quartet" (International Diabetes Federation, 2003). According to the World Health Organisation, moderate hypertension is classified as systolic pressure ≥160 mmHg and diastolic pressure ≥90-100 mm Hg (Joint National Committee on prevention, 1997). A study in a group of patients with newly diagnosed type 2 diabetes, showed that 39% had hypertension (The Hypertension in Diabetes Study Group, 1993).

Obesity manifests as an increased body mass index (BMI) greater than 30, calculated as weight in kilograms divided by the square of height in metres. Obesity predisposes to the development of type 2 diabetes as do frequent and large meals, although the exact mechanisms for this are unknown (Mokdad *et al.*, 2003). An epidemiological study has shown that patients with obesity have a 30-40 fold increased risk of type 2 diabetes compared to the risk of developing other diseases (Field *et al.*, 2001). Obesity is not a risk factor for type 1 diabetes (Adler ^002) however, subjects with type 1 diabetes who are obese have an increased risk of cardiovascular disease (Idzior-Walus *et al.*, 2001).

The most common form of dyslipidemia in type 2 diabetes is hypertriglyceridemia with increased very-low-density lipoprotein (VLDL)-cholesterol, decreased high-density lipoprotein (HDL)-cholesterol (Gaw *et al.*, 1995) and the appearance of small dense LDL (O'Brien *et al.*, 1997). The United Kingdom Prospective Diabetes Study (UKPDS) has shown that triglyceride levels are 50% higher in patients with type 2 diabetes compared to normal subjects (UK Prospective Diabetes Study Group, 1997). Additionally multiple clinical studies have found that hypertriglyceridemia increases the risk of cardiovascular disease in diabetes (Fontbonne *et al.*, 1989; Manson *et al.*, 1991; Stamler *et al.*, 1993). Biochemically, a high level of triglycerides (TGs) leads to an increase in TG-degradation products, called free fatty acids (FFAs). Free fatty acids are elevated in subjects with type 2 diabetes (Reaven *et al.*, 1988) and have been shown to inhibit the effect of insulin to stimulate glucose uptake in patients with and without type 2 diabetes (Boden & Chen, 1995). Additionally, chronically elevated FFAs decrease pancreatic β-cell function to secrete insulin and reduce β-cell mass by inducing apoptosis (Cnop *et al.*, 2001; Lupi *et al.*, 2002).
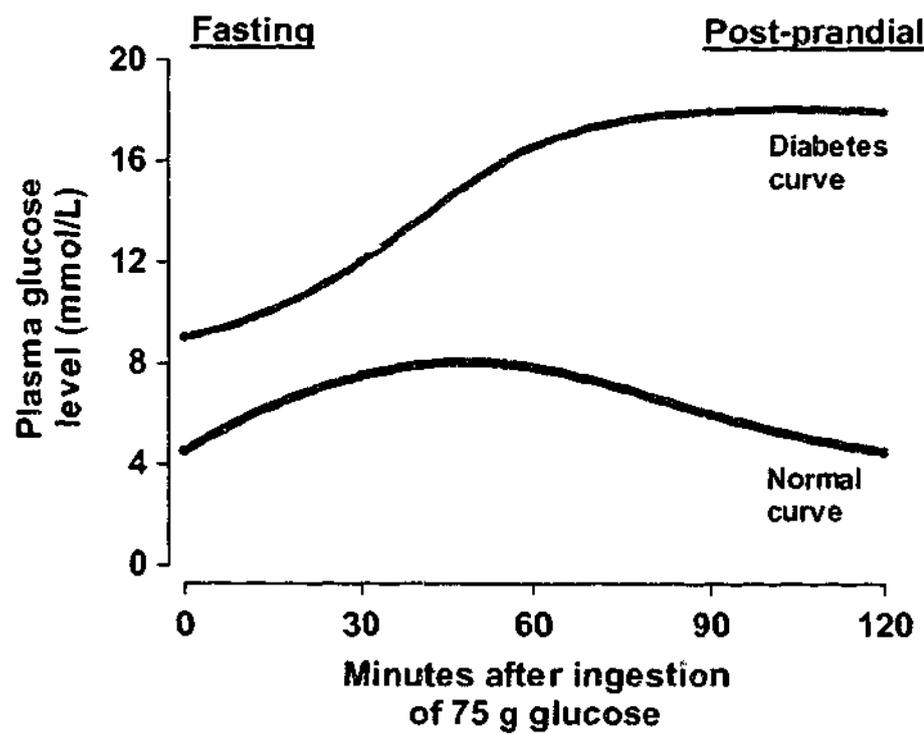
Figure 1-1

Plasma glucose levels following an oral glucose load. Fasting plasma glucose concentration is higher in subjects with diabetes than for normal subjects. Following administration of a glucose load (75 g), plasma glucose returns to fasting glucose plasma concentrations within 2 h in normal subjects, but remains elevated for longer periods of time, in patients with diabetes. Normal subjects (blue line) and patients with diabetes (red line). Modified from (Gaw *et al.*, 1995).

1.1.3 Treatment and prognosis

Both type 2 diabetes and obesity are potentially prevented and partially controlled by lifestyle modifications. This includes both a change in diet (reduced intake of fat and saturated fat, increased intake of fibre) and an increase in physical activity with an aim of achieving a BMI <25. A clinical study in type 2 diabetes high risk patients has shown that modification of diet and increased physical activity were superior to glucose lowering therapy in reducing the incidence of type 2 diabetes (Knowler *et al.*, 2002). Recent results from the Finnish Diabetes Prevention Study have shown that intensive lifestyle changes (diet and exercise) reduce the risk of developing diabetes by 58% ($P<0.001$) in obese patients with impaired glucose tolerance (Tuomilehto *et al.*, 2001). In patients with diagnosed type 2 diabetes, an exercise regime of three 55 min sessions per week is enough to reduce the need for direct insulin therapy (Dunstan *et al.*, 2002a).

Therapeutic indications for the prevention of atherosclerosis in diabetes clearly emphasize a multi-factorial approach to achieve tight control of hyperglycemia, hypertension, and dyslipidemia in addition to avoiding cigarette smoking and limiting obesity (Shantaram, 1999). For controlling blood glucose, sulfonylureas and biguanides are usually prescribed. These drugs act by increasing the ability of tissues to take up and store glucose or increase the release of glucose from the pancreas. A new class of medicines has recently been introduced for the treatment of hyperglycemia. Insulin sensitizers such as glitazones are currently being used with success in many countries; their action is to increase the responsiveness of tissues to insulin. If the patient's hyperglycemia is unresponsive to lifestyle changes and oral pharmacological measures, the use of direct insulin injection is required.

Both the UKPDS (UK Prospective Diabetes Study Group, 1998b) for type 2 diabetes and the Diabetes Control and Complications Trial (The Diabetes Control and Complications Trial

Research Group, 1993) for type 1 diabetes have concluded that the pharmacological control of plasma glucose alone by insulin may be sufficient to prevent microvascular complications but not macrovascular complications associated with diabetes. The treatment of hypertension in diabetes with both a β-blocker and an angiotensin converting enzyme (ACE) inhibitor, reduces the risk of developing all diabetes related complications by 24% and all macrovascular diseases by 34% (P=0.019) (UK Prospective Diabetes Study Group, 1998c). Combination therapy with an ACE inhibitor to control blood pressure (UK Prospective Diabetes Study Group, 1998a), and a statin or a fibrate for controlling dyslipidemia is strongly recommended for patients who have an increased coronary risk (Barter, 2001; Zimmet *et al.*, 2003). Fibrates lower triglycerides and have the additional benefit of moderately increasing HDL (Rubins *et al.*, 1999), and these corrections in lipid profile are usually required in patients with type 2 diabetes. Additionally, in human subjects and animal models with diabetes, treatment with fibrates or fibrate-like agents reduces FFAs and improves insulin sensitivity of muscle tissue (Damci *et al.*, 2003; Ye *et al.*, 2001).

A patient with either type 1 or type 2 diabetes will not usually die from the disease itself, but from one of its many vascular complications (Figure 1-2). The prognosis for diabetes is poor for patients with type 1 diabetes showing a 3 to 6 fold increase in mortality (Muller, 1998) compared to normal subjects, and fatal complications may occur from 15-25 years after onset (Vardaxis, 1994). The most likely cause of death in type 1 diabetes is end stage renal disease and vascular disease, however advances in treatment have shown that deteriorating renal function is improved with strict control of blood pressure (Mogensen *et al.*, 1995; Parving *et al.*, 1987). Mortality of subjects with type 2 diabetes is 1.7-3.4 times that of normal subjects (Muller, 1998). Life expectancy is dependent on the stage of detection, the presence of risk factors, compliance of patients with therapeutic treatment/lifestyle modifications and

development of vascular complications (Gaede *et al.*, 2003). The major causes of death in type 2 diabetes are myocardial infarction (50% of cases), cerebrovascular events (12% of cases), renal failure (12% of cases) and various cancers (10% of cases) (Vardaxis, 1994). Congestive heart failure and gangrene, a result of lower limb vascular disease, are also common causes of death. The burden of debilitating vascular complications on the individual and the health services highlights the need for aggressive management of diabetes and its associated vascular disease.

Microvascular disease   **Macrovascular disease**
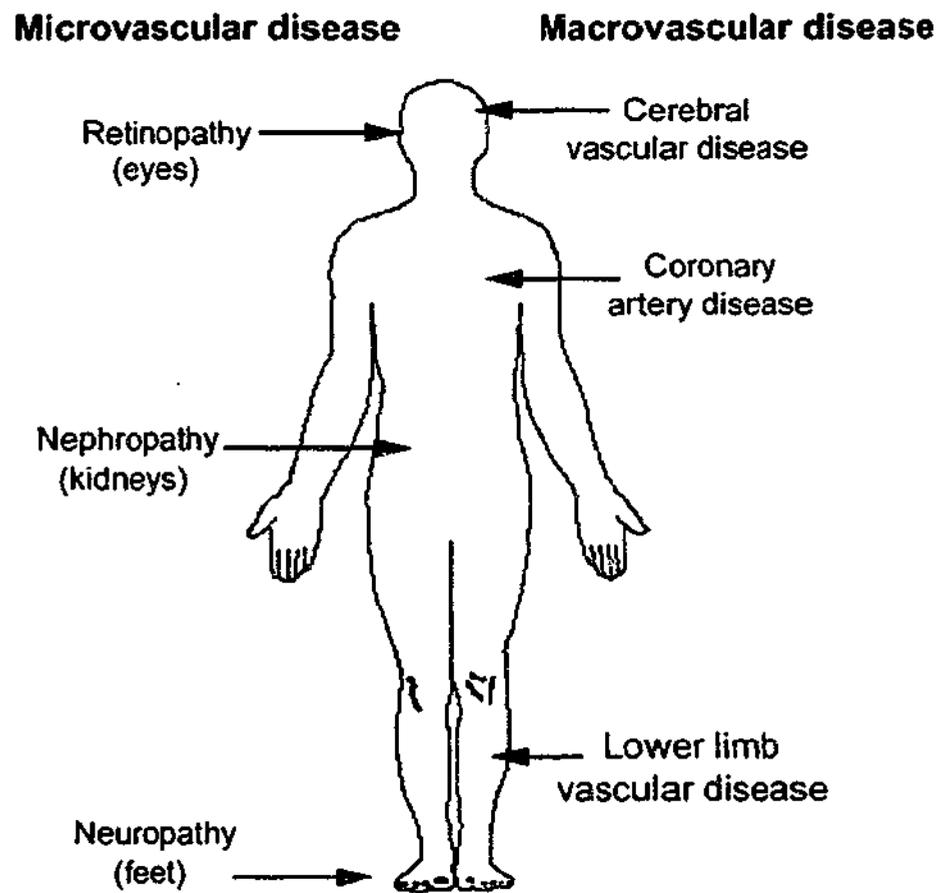
Figure 1-2

Summary of macrovascular and microvascular complications of diabetes. Microvascular disease destroys the small blood vessels and capillaries in the eyes, kidney and the blood vessels which supply the nerves in the feet. Macrovascular disease affects large-medium sized blood vessels in the brain (cerebral vascular disease) heart (coronary artery disease) and the lower limbs. Adapted from (Bate & Jerums, 2003; Vardaxis, 1994).

1.2 Triglyceride lowering agents

1.2.1 Discovery and history

The first lipid lowering agent known as a fibric acid derivative was chemically synthesized by Thorp and Waring in 1962 (Thorp & Waring, 1962) and was called clofibrate. While having modest effects on improving lipid abnormalities, a World Health Organization Cooperative Trial in 1978 and 1980, showed that clofibrate increased mortality from ischemic heart disease, stroke, and cancer (Report from the Committee of Principal Investigators, 1978, 1980). The use of clofibrate was restricted and it is no longer available in Australia. A number of structurally related compounds were synthesized in a bid to increase the specificity and lipid-lowering potency. Methylclofenapate had an additional benzene ring but was less active than the parent compound. Fournier Laboratories synthesized a similar compound with a para-chlorine and an isopropyl side chain (Kloer, 1987), which was then called procetofen and is now known as fenofibrate. Other fibric acid derivatives that have been clinically used for controlling hypertriglyceridemia include bezafibrate and gemfibrozil. Gemfibrozil is widely used in Australia while fenofibrate is used in the US. Fenofibrate is the subject of the Fenofibrate and Event Lowering in Diabetes (FIELD) study, which involves patients with type 2 diabetes from Australia, New Zealand and Finland.

In 1990, Isseman and Green (Issemann & Green, 1990) recognized that the fibrate compounds were ligands for the peroxisome proliferating-activated receptor (PPAR)-α, a nuclear receptor that regulates the expression of multiple genes including those involved in lipid metabolism. Since the description of PPAR-α more potent PPAR-α ligands have been synthesized as research tools such as WY-14,643 and GW7647, a ureido-fibrate (Brown *et al.*, 2001) but neither of these have become clinically available.

1.2.2 Structure and Clinical Pharmacology

Fenofibrate is a phenoxyisobutyric acid derivative, similar to other fibric acid derivatives, gemfibrozil, bezafibrate and clofibrate (Figure 1-3). Fenofibrate is a pro-drug which is rapidly hydrolyzed by tissue and plasma esterases to its active metabolite, fenofibric acid (Balfour *et al.*, 1990). Studies of plasma concentration of fenofibrate have indicated peak plasma concentrations of 16 to 28 μmol/L, reached after 4-6 hours of administration of 300 mg dose (Balfour *et al.*, 1990). The peak plasma concentration of gemfibrozil (80 μmol/L) occurs 1-2 hours after administration (Zimetbaum *et al.*, 1991). PPAR-α ligands used as research tools such as WY-14,643 and GW7647, a urea substituted thioisobutyric acid, show a higher affinity for PPAR-α than the clinically used fibrates (Figure 1-3 and Table 1-1).

Clofibrate

Bezafibrate   **Gemfibrozil**

Clinically
used
agents

Fenofibrate

WY-14,643   **GW7647**
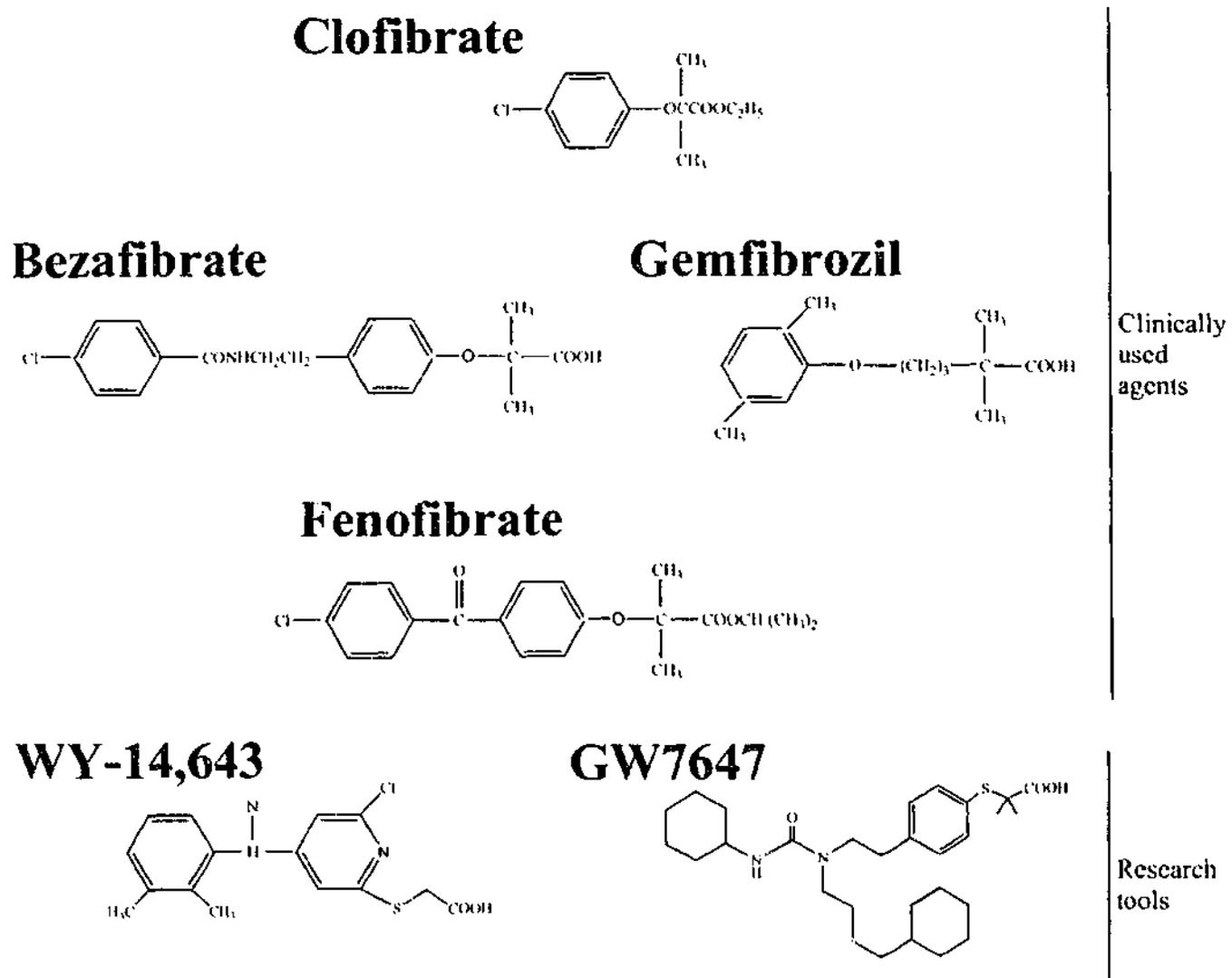
Research
tools

Figure 1-3

Chemical structures of clinically used fibrates and other PPAR-α ligands used as research tools. Adapted from (Brown *et al.*, 2001).

Compound	EC$_{50}$ (μmol/L)		
	Human PPAR-α	Human PPAR-γ	Human PPAR-δ
Fenofibric acid	30	300	Inactive at 100
Bezafibrate	50	60	20
Clofibric acid	55	$\approx$500	Inactive at 100
WY-14,643	5	60	35
GW7647	0.006	1.1	6.2
Troglitazone	Inactive at 10	0.55	Inactive at 10
Rosiglitazone	Inactive at 10	0.043	Inactive at 10
Pioglitazone	Inactive at 10	0.58	Inactive at 10

Table 1-1

Affinity of fibrates, PPAR-α ligand research tools and glitazones for the human PPAR subtypes *in vitro*. The fibrates (fenofibric acid, bezafibrate and clofibirc acid) are PPAR-α ligands however at high concentrations they can activate PPAR-γ. The PPAR-α ligand research tools, WY-14,643 and GW7647 are 6-fold and 5000-fold more potent ligands for PPAR-α than fenofibric acid, respectively. The glitazones (troglitazone, rosiglitazone and pioglitazone) are specific ligands for PPAR-γ. Adapted from (Brown *et al.*, 2001; Willson *et al.*, 2000).

1.2.3 Mechanism of action of fibrates for treating hypertriglyceridemia

Gemfibrozil and fenofibrate are effective in decreasing elevated triglycerides and moderately decrease elevated LDL-cholesterol. These compounds also have an important and uncommon ability to increase HDL-cholesterol levels. The precise mechanism of action of fibrates on lipid metabolism is not clear because they have a wide range of effects and often it is unknown which are direct effects of the drug and which are secondary effects. The target genes of the activated receptor on which the fibrates act (PPAR-α) are mostly associated with lipid catabolism but the fibrates also interfere with lipoprotein synthetic pathways.

1.2.3.1 Effects of fibrates on the synthetic lipoprotein pathway

In the endogenous lipoprotein pathway, cholesterol and triglycerides are synthesized in the liver and transported as VLDL (30-80 nm diameter particles) to muscle and adipose tissue where triglycerides are hydrolyzed and the free fatty acids are taken up by tissues. Fenofibrate inhibits the de novo synthesis of fatty acids in the liver as a consequence of the inhibition of lipolysis in adipose tissue, reducing the amount of fatty acid available for triglyceride synthesis (Kritchevsky *et al.*, 1979). In various animal models, fenofibrate has been shown to interfere with *de novo* triglyceride metabolism by increasing mitochondrial free fatty acid uptake and fatty acid oxidation through stimulation of muscle-type carnitine palmoyltransferase (CPT-1) in skeletal and cardiac muscle (Kloer, 1987). The moderate effects of fibrates to increase HDL-cholesterol are attributable to the PPAR-α regulated increased expression of HDL major apolipoproteins, apolipoprotein A-I and A-II.

1.2.3.2   *Effects of fibrates on the catabolic lipoprotein pathway*

The catabolism of triglycerides is controlled by the enzyme, lipoprotein lipase (LPL), which is found on the endothelium of capillaries in skeletal and adipose tissues (Kaplan & Pesce, 1996; Kloer, 1987; Rang *et al.*, 1995). Triglycerides are hydrolysed by LPL, releasing fatty acids, which are taken up by surrounding tissue, and chylomicron remnants which are passed to the liver and undergo endocytosis. Lipoprotein lipase is activated by apolipoprotein CII and inhibited by apolipoprotein CIII and a change in the plasma levels of these two apolipoproteins modulates the catabolism of chylomicrons and VLDL. Fenofibrate has been shown to inhibit apolipoprotein CIII expression thereby increasing the ratio of CII to CIII which enhances LPL activity and improves the capacity for triglyceride removal (Franceschini *et al.*, 1985; Kloer, 1987).

1.2.4   Clinical trials with fibrates

Clinical treatment of patients with fenofibrate began in 1975 (Roberts, 1989). The evidence that fibrates have cardioprotective actions in diabetes has been slow to emerge because of the general exclusion of this group of patients in the early trials or the disease being overlooked. The 12 trials published between 1966 and 1996, included therapy with fibrates or placebo in more than 21,000 patients. Overall, these trials did not indicate that fibrate treatment reduced the risk of coronary related death, possibly because of the exclusion of "high risk" patients. The trials conducted since 1996 to date, have mostly concluded with positive evidence for the benefits of fibrates in reducing end points of angiographic progression or major cardiovascular events.

The Helsinki Heart Study (HHS) was a controlled primary prevention trial to test the hypothesis that using gemfibrozil to lower serum LDL and VLDL and to raise HDL protects

subjects against coronary heart disease. The trial had 4,081 middle aged male subjects, 135 of whom had type 2 diabetes. As expected after five years of treatment, gemfibrozil improved the lipid profiles by increasing HDL-cholesterol by 11% and decreasing triglycerides and LDL-cholesterol by 35% and 11%, respectively (Huttunen *et al.*, 1991). A follow-up analysis of the subjects with diabetes in the HHS showed that although the lipid changes were similar in patients with diabetes versus patients without diabetes, the patients with diabetes had a lower incidence of coronary heart disease (CHD) with gemfibrozil treatment (3.4%, n=59) compared to patients with diabetes in the placebo group (10.5%, n=76) although this did not reach statistical significance (Koskinen *et al.*, 1992).

The Veterans Affairs-HDL Intervention Trial (VA-HIT), involving the treatment of male subjects with gemfibrozil over 5 years, had a positive outcome. The study showed that there was a significant reduction of 22% (P=0.006) in the combined incidence of fatal and non-fatal coronary events, and a significant reduction (24%, *P*<0.001) in deaths from coronary heart disease, non-fatal myocardial infarction and stroke (Rubins *et al.*, 1999).

The results of the Bezafibrate Infarction Prevention (BIP) Trial were negative because they demonstrated no significant effect of treatment with bezafibrate on either the primary end-point of the trial or on rates of coronary death. Conversely, bezafibrate treatment was beneficial in the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT), a small study with 81 young male subjects with previous myocardial infarction (Ericsson *et al.*, 1996). Bezafibrate treatment in these patients showed a significant reduction in the extent of narrowing of segmental lesions that comprised 20-50% of the coronary lumen. The Lopid Coronary Angiography Trial (LOCAT) involved the use of gemfibrozil in male subjects with low HDL cholesterol as their main lipid abnormality who had undergone coronary artery by-pass grafting (CABG) (Frick *et al.*, 1997). The study showed a significant reduction in the

formation of bypass-graft lesions after coronary bypass surgery with gemfibrozil treatment.

The Diabetes Atherosclerosis Intervention Study (DAIS) is the only completed trial to date, which specifically looked at fibrate treatment in diabetes. Subjects with type 2 diabetes (n=418, 305 men, 115 women) with at least one coronary lesion were randomized to fenofibrate or placebo for 3 years. The results showed a 29% and 7% decrease in triglycerides and LDL-cholesterol respectively and a 6% increase in HDL-cholesterol. In addition, fenofibrate treatment significantly reduced the progression of coronary narrowing by 40% (Diabetes Atherosclerosis Intervention Study Investigators, 2001; Faergeman, 2000; Steiner, 2000).

Although the recent clinical trials have demonstrated that fibrates are beneficial for the prevention of CHD, the fibrates have not been satisfactorily defined by the randomized clinical trials published to date. Ongoing trials will clarify the beneficial effects of fibrate treatment in patients with type 2 diabetes including the FIELD Study currently being conducted in patients with diabetes from Australia, New Zealand and Finland and is scheduled to conclude in 2004-5. This trial will determine whether fenofibrate has beneficial effects on the macrovascular complications of diabetes (Despres, 2001; Steiner, 2000).

1.3   Peroxisome Proliferating-Activated Receptors (PPARs)

1.3.1   Function and Distribution

Peroxisome Proliferating-Activated Receptors (PPARs) are a group of nuclear receptors controlling fatty acid metabolism and storage (Schoonjans et al., 1996). PPAR-α was the first PPAR to be identified and characterized in mouse liver, by Isseman and Green in 1990 (Issemann & Green, 1990). Soon after PPAR-α was shown to be expressed in human tissues (Mukherjee et al., 1994; Sher et al., 1993). Two other isoforms of PPAR have been identified

in animals and humans. PPAR-γ (Kliewer *et al.*, 1994; Zhu *et al.*, 1993) and PPAR-δ (Schmidt *et al.*, 1992). The -α and -γ isoforms are concentrated in different tissues (Table 1-2). PPAR-α is mostly found in tissues with high metabolic activity such as the liver, kidney, heart and muscle and regulates genes involved in fatty acid metabolism (Kliewer *et al.*, 2001). PPAR-γ is mostly distributed in the adipose tissue and its primary role is to control adipocyte differentiation (Tontonoz *et al.*, 1994) and regulate genes involved in glucose and lipid homeostasis (Willson *et al.*, 2000). PPAR-δ is ubiquitously expressed and its biological function is somewhat unclear however, the recent synthesis of a specific PPAR-δ ligand, GW1516, suggests that like PPAR-α, this receptor is involved in reducing triglycerides and increasing HDL-cholesterol (Oliver *et al.*, 2001). Various PPAR isoforms are also expressed in cells of the vasculature such as endothelial cells (Marx *et al.*, 1999) and smooth muscle cells (Law *et al.*, 2000; Staels *et al.*, 1998a), and inflammatory cells such as monocytes/macrophages (Kintscher *et al.*, 2000; Marx *et al.*, 1998) and T lymphocytes (Marx *et al.*, 2002) where they have a role in mediating the inflammatory processes associated with atherosclerosis (Li & Glass, 2002; Plutzky, 2001, 2003). The vascular actions of PPAR-α are reviewed further in Section 1.8.1.

1.3.2 Mechanism of action

In the presence of a pharmacological PPAR ligand such as fenofibrate (alpha) or troglitazone (gamma) or a natural ligand (fatty acids and prostaglandins) (Neve *et al.*, 2000; Willson *et al.*, 2000), PPARs translocate from the cytoplasm to the nucleus where they recruit the retinoic acid receptor (RxR) and its ligand, 9-cis retinoic acid. Together, these two receptors heterodimerize and bind to the PPAR response element (PPRE) of multiple genes to modulate transcription (Figure 1-4). Hence, PPARs exert their activity directly within a cell's

nucleus through transactivation (gene stimulation) (Neve *et al.*, 2000). Additionally the PPARs can influence transrepression (gene inhibition) by indirectly antagonizing nuclear factor-κB (NF-κB) and activator protein (AP)-1 pathways through a DNA-binding independent mechanism (Figure 1-4) (Chawla *et al.*, 2001; Cunard *et al.*, 2002). NF-κB and AP-1 are transcription factors which modulate the activity of proinflammatory cytokines (e.g. interleukin-1β). Transrepression is the mechanism by which PPAR ligands may exert anti-inflammatory actions in atherosclerosis (Staels *et al.*, 1998b). It has recently been suggested that PPAR-δ may control both pro- and anti-inflammatory responses in atherosclerosis (Lee *et al.*, 2003; Plutzky, 2003).

		Ligands		
Isoform	Tissue	Endogenous	Synthetic	Result of stimulation
PPAR-α	Liver	8(S)hydroxyeicosatetraenoic	Fibric acid	Modulates genes
	Heart	acid	derivatives e.g.	involved in lipid and
	Kidney	8(S)hydroxyeicosapentaenoic	Gemfibrozil,	lipoprotein metabolism
	Intestinal mucosa	acid	fenofibrate,	
	Brown adipose		WY-14,643	
	Vascular SMCs			
PPAR-γ	Adipose tissue	15-deoxy-$\Delta^{12,14}$	Thiazolidinediones	Cellular differentiation;
	Vascular SMCs	Prostaglandin J_2	e.g. rosiglitazone	Regulation of
				adipogenesis and
				insulin action
PPAR-δ	Ubiquitous	Unknown	GW1516	$\downarrow$ triglycerides
				$\uparrow$ HDL-cholesterol

Table 1-2

Summary of tissue localization, natural and chemical ligands and result of stimulation of PPAR-α, PPAR-γ and PPAR-δ. Adapted from (Law et al., 2000; Oliver et al., 2001; Pineda Torra et al., 1999; Staels et al., 1998a).
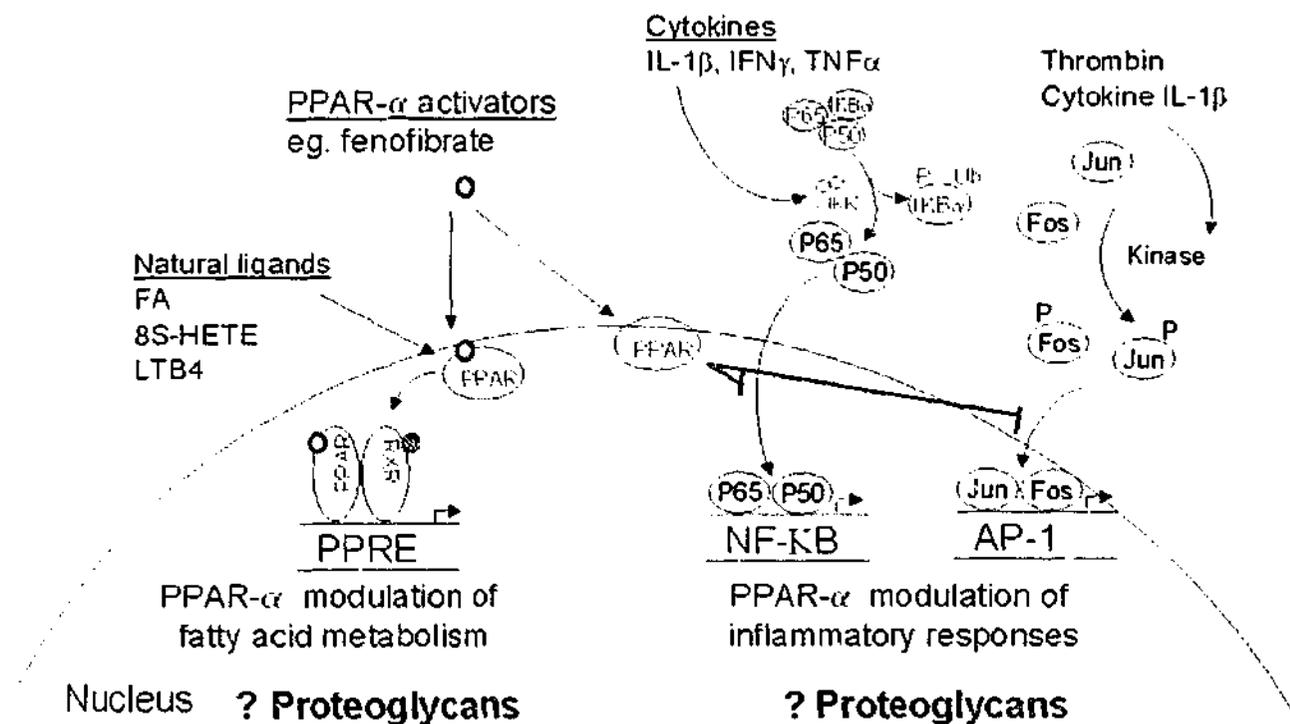
Figure 1-4

Mechanism of action of PPAR-α ligands. PPARs exert their activity directly within a cell's

nucleus through transactivation (gene stimulation). PPARs can influence transrepression (gene

inhibition) by indirectly antagonizing nuclear factor-κB (NF-κB) and activator protein (AP)-1

pathways through a DNA-binding independent mechanism. Adapted from (Neve *et al.*, 2000).

1.3.3 Role of PPARs in lipid metabolism

The role of PPARs in lipid metabolism is related to the effects of fibrates on lipid metabolism detailed under Section 1.2.3. The effects of PPAR-α ligands to control dyslipidemia may involve the transactivation of PPREs on the lipoprotein lipase, liver-fatty acid binding protein, cytochrome P450 and acyl-CoA oxidase genes. A recent study has shown that an alteration in the PPAR-α gene exacerbates the dyslipidemia in type 2 diabetes but not in normal patients and could be a link between type 2 diabetes and increased risk of coronary artery disease (Flavell *et al.*, 2000).

Ligands for PPAR-α, PPAR-γ and PPAR-δ have been shown to have varying degrees of effectiveness in controlling serum lipids, with the order of effectiveness being α>γ>δ (Vosper *et al.*, 2002). The ligands for PPAR-α are not totally specific and there is some cross over onto other isoforms (Table 1-1). As seen with the administration of ACE inhibitors for controlling blood pressure (UK Prospective Diabetes Study Group, 1998a), PPAR-α ligands used in patients with diabetes are as effective in normalising dyslipidemia as in patients without diabetes (de Salcedo *et al.*, 1976; Steiner, 2000).

1.4 The cardiovascular system in diabetes

The cardiovascular system is comprised of the heart and all of the blood vessels involved in the transport of blood to and from the heart. Arteries are muscular and innervated vessels that pump blood from the heart to the major organs and tissues throughout the body. Within tissues, nutrients and oxygen are supplied by diffusion through the lumen of small arteries and capillaries (monocell-layered vessels). Veins are thin walled vessels that carry de-oxygenated blood from the tissues back to the heart with the assistance of venous valves (Tortora & Grabowski, 1996). The two major blood vessels supplying blood to the heart itself (coronary

arteries) branch out from the ascending aorta, and are the left coronary artery and the right coronary artery. The left coronary artery passes inferior to the left atrium and divides into the left anterior descending (LAD) artery and circumflex branch (Figure 1-5) (Tortora & Grabowski, 1996). The LAD supplies oxygenated blood to the walls of both ventricles and the interventricular septum (inside the heart). The circumflex branch supplies oxygenated blood to the left ventricle and left atrium. The right coronary artery has small branches which supply the right atrium. The right coronary artery lies inferior to the right ventricle and divides into the posterior interventricular branch which distributes oxygenated blood to both ventricles and the interventricular septum, and the marginal branch which supplies the myocardium of the right ventricle (Figure 1-5).

1.4.1   Physiological structure of large and medium-sized arteries

A large artery is composed of 3 main layers, 1) the innermost layer which is called the intima and is comprised of a monolayer of endothelial cells and the internal elastic lamina (IEL) 2) the bulk of the vessel is the medial layer comprising 4-8 layers of smooth muscle cells which have a contractile phenotype (Campbell et al., 1987) organized within a meshwork of collagen, elastic fibres, glycoproteins and proteoglycans and 3) an adventitia layer which is separated from the media layer by the external elastic lamina and is composed of loosely arranged fibroblasts, collagen and elastic fibres, (di Fiore, 1988), is rich in lymphatics and traversed by nerves which supply the medial smooth muscle (Figure 1-6). The adventitia of large vessels also has a vasa vasorum, which nourishes the adventitia and outer media layer, while the inner layers are supplied with nutrients by diffusion from the lumen (Muir, 1992).
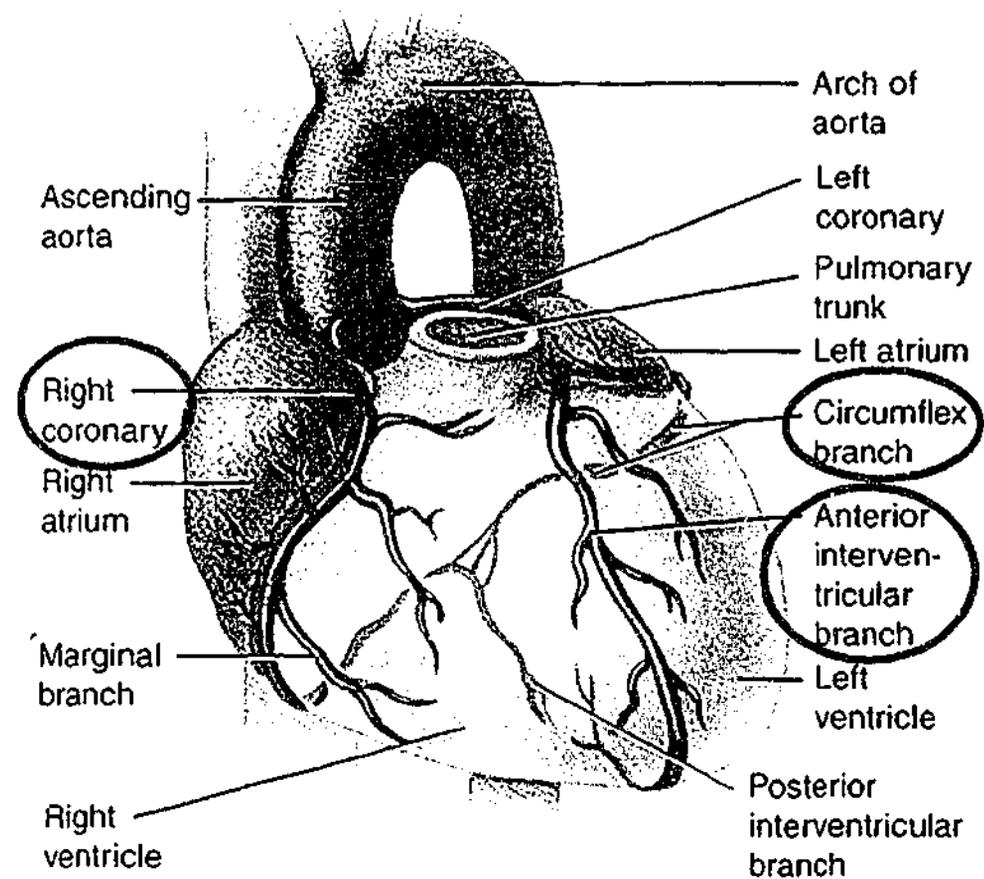
Figure 1-5

Major blood vessels of the heart. The right coronary artery, circumflex branch and the anterior interventricular branch (left anterior descending artery) are most susceptible to developing atherosclerosis and are circled in red. Adapted from (Tortora & Grabowski, 1996).

Adventitia

Internal elastic lamina

Endothelium

External elastic lamina

Media layer
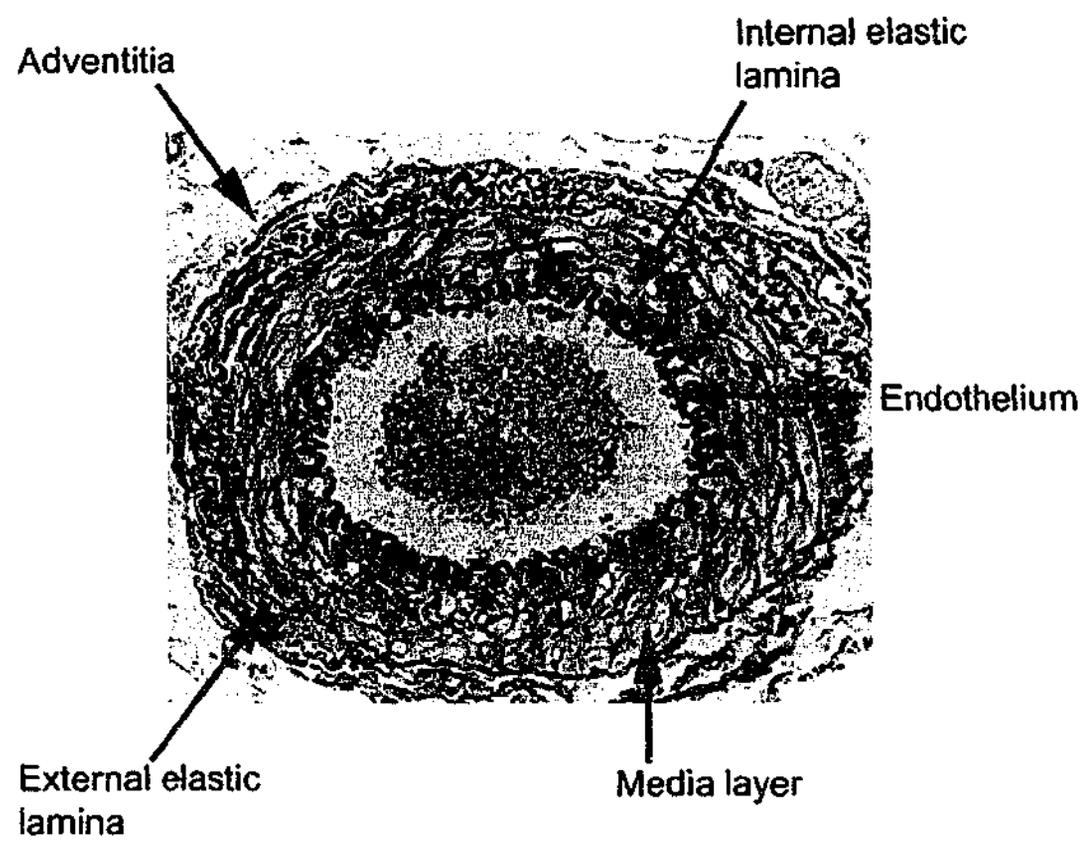
Figure 1-6

Histological section of a normal medium sized artery. Transverse section × 10, from (Muir, 1992).

1.4.2 Pathology of atherosclerosis

Cardiovascular disease is the largest cause of death in Western societies and it primarily results from atherosclerosis of the coronary arteries leading to myocardial infarction when it occurs in the coronary arteries or stroke when it occurs in the cerebral arteries (Braunwald, 1997; Haffner *et al.*, 1999). Both type 1 and type 2 diabetes lead to an increased rate of atherosclerosis with sudden death from myocardial infarction or delayed death from heart failure (Balkau *et al.*, 1997; Kannel & McGee, 1979, Laakso & Lehto, 1998), however the exact mechanisms for the increased susceptibility and progression of atherosclerosis in patients with diabetes are unknown (Gaw *et al.*, 1995).

The most common site of atherosclerosis of the coronary vessels is the LAD coronary artery (50% of MI), followed by the main trunk on the right (30% of MI) and the left circumflex branch (20% of MI) (Figure 1-5) (Vardaxis, 1994). Rupture of a plaque and occlusion of the LAD leads to infarction of sections of the left ventricle, with the right ventricle and atria sections of the heart rarely being affected (Muir, 1992; Vardaxis, 1994). The physical positioning of the coronary vessels and their exposure to high mechanical pressures may exacerbate coronary narrowing, plaque formation and eventually occlusion. The area of myocardial infarction is dependent on the coronary vessel(s) affected by atheroma. Small infarcts are confined to a central subendocardial or subpericardial area while a transmural infarct affects the whole thickness of the left ventricle affected (Muir, 1992; Vardaxis, 1994). The infarcted area of the cardiac tissue undergoes necrosis and is eventually replaced with fibrous tissue, which places added strain on the heart to maintain its normal cardiac output.

Macroscopically, the wall of the atherosclerotic artery is thickened, has lost its elasticity and the lumen diameter is reduced. The earliest deposit of lipid in the intima is called a fatty

streak which appears as a yellow, slightly raised area on the luminal surface which enlarges and comes together to form irregular yellow streaks. The fatty streak contains accumulations of lipid droplets beneath the endothelium that are free and within macrophages. The fatty streak occurs in childhood and adolescence (McGill *et al.*, 2000; Ross, 1993; Stary *et al.*, 1994). The proceeding lesion contains gelatinous patches caused by oedema and an increase in proteoglycans. Intimal cushions are observed at branching points of arteries due to intimal thickening and are composed of smooth muscle cells (SMCs), collagen and other extracellular matrix proteins (Muir, 1992). The atherosclerotic plaque appears as a small, disc-like, slightly raised patch of intimal thickening with a smooth glistening surface, appearing yellow (lipid deposits) and white due to the superficial fibrous tissue. A complicated plaque is one that has cracked or ulcerated, and a thrombus has been deposited on the surface or shows calcification which converts the lesion to a hard brittle plate (Berliner *et al.*, 1995; Shanahan *et al.*, 1994).

Microscopically the atherosclerotic vessel appears as a thickened intima due to the proliferation of smooth muscle cells and the accumulation of lipids in foam cells. Thin strands of connective tissue develop beneath the endothelium and between the foam cells. Areas of necrosis develop in the deeper part of the lesion, converting it to a structureless accumulation of extracellular lipid, cholesterol crystals and tissue debris as well as neutrophils and other inflammatory cells (Muir, 1992). An intermediate lesion is composed of layers of macrophages and SMCs (Ross, 1993) and is likely to advance to a complex and occlusive lesion, which is identified as a fibrous plaque. As the plaque thickens, the media becomes thin and weak in an attempt to structurally dilate and maintain patency ("remodelling") and the lesion may erode into the media by disrupting the internal elastic lamina (Figure 1-7). The plaque may rupture causing haemorrhage into the plaque, leading to the formation of a thrombus (Muir, 1992). The thrombus may partially or totally occlude the lumen leading to

clinical events such as myocardial infarction, stroke or limb amputation.

Atherosclerotic plaque

Partially obstructed lumen
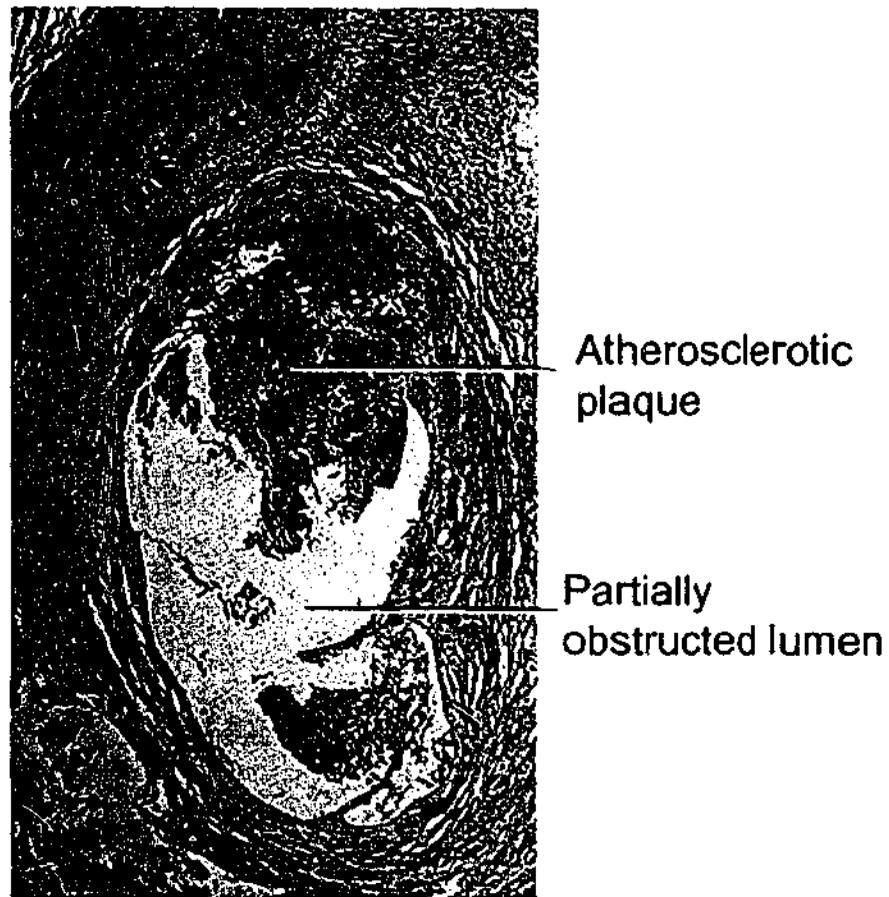
Figure 1-7

Accumulated cholesterol, triglyceride and cells form an atherosclerotic plaque.

Transverse section of a coronary artery from (Tortora & Grabowski, 1996).

1.4.2.1 Hypotheses of atherogenesis

Atherosclerosis is not a disease in its own right, but a process which is the major contributor to myocardial and cerebral infarction, gangrene and loss of function in the extremities (Ross, 1993). Over the last century, various hypotheses have been forwarded to explain the initiating events and factors which contribute to the development of atherosclerosis. There are three linked hypotheses of atherogenesis which have numerous components and they include the "response-to-injury" hypothesis, "response to retention" hypothesis and the "oxidation hypothesis".

Russell Ross (Ross & Glomset, 1976a, 1976b) is well regarded for his annunciation of the "response-to-injury" hypothesis, which states that endothelial damage precedes smooth muscle cell migration and proliferation, deposition of intracellular and extracellular lipid, and accumulation of extracellular matrix components including proteoglycans. More recently the "response-to-injury" hypothesis was broadened to include endothelial dysfunction as a key event which initiates the inflammatory mechanisms associated with atherosclerosis (Ross, 1999).

In 1995, the work of various groups was annunciated by Kevin J. Williams and Ira Tabas (Williams & Tabas, 1995, 1998) as the "response to retention" hypothesis which proposes that the central atherogenic process is the sub-endothelial retention and accumulation of lipoproteins, by extracellular matrix molecules, such as proteoglycans. The importance of this hypothesis was strongly supported by the work of Jan Boren and colleagues (Skalen *et al.*, 2002) who showed that mice expressing genetically modified ApoB that had defective binding of LDL to proteoglycans developed atherosclerosis at a rate that was appreciably less than those mice expressing normal Apo B and thus normal LDL-proteoglycan binding.

The "oxidation hypothesis" of atherosclerosis was affirmed by Joseph L. Witzum in 1994

(Witztum, 1994). The central component of the "oxidation hypothesis" is the oxidative modification of LDL, which acts as an immunogenic stimulus for monocyte recruitment to the vessel and phagocytic uptake of oxidized-LDL by macrophages. The process of oxidation of cellular components and particles such as lipoproteins is thought to exacerbate atherosclerosis, although this is generally a feature of late stage lesions (Griendling & Alexander, 1997; Witztum, 1994).

Of the three hypotheses, the "response-to-retention" hypothesis is thought to best describe the key initiating event in atherogenesis: LDL retention by vascular proteoglycans. We recognized the possibility that the "response-to-retention" hypothesis would serve as a model to study potential therapeutic interventions of proteoglycan-LDL binding. The ability to alter vascular proteoglycans may represent an important route for therapeutic intervention to prevent cardiovascular disease hence, the studies presented in this thesis are primarily founded on the "response-to-retention" hypothesis.

1.4.2.2   Aetiology and risk factors for atherosclerosis

Atherosclerosis arises from an interaction between blood-borne lipoproteins and pathological events occurring within blood vessels. The non-modifiable risk factors associated with developing atherosclerosis include genetic predisposition (Hegele, 1999; Marian, 1997), gender effects (Shantaram, 1999; Watarai et al., 1999) and age (Merat et al., 2000).

A familial study in identical and non-identical twins has shown that if one identical twin died from coronary artery disease at a young age (36-65 years) the other had a risk of death that was three times greater however, this relationship was not maintained in older twins (Marenberg et al., 1994). Another report has shown that there is a 40% risk of macrovascular disease in families with type 2 diabetes (Marenberg et al., 1994). These findings suggest that

there is a genetic mechanism underlying atherogenesis however, because atherogenesis is a complex pathological process involving multiple cells and stimuli, it is probable that there are multiple genes involved in regulating atherogenesis (Marian, 1997). The recent sequencing of the human genome may allow for identification of polygenetic variations in atherosclerosis-susceptible subjects, such as people with diabetes.

There is a lower age-specific incidence of coronary heart disease in women however, after the age of 65 years the risk of coronary artery disease surpasses men by 11% (Eaker *et al.*, 1993). These observations suggest that post-menopausal females may lose the cardioprotective effects attributed to oestrogen (Kannel & Levy, 2004). Studies have shown that oestrogen has favourable effects on the vasculature including increasing endothelial nitric oxide synthase, which causes vasodilatation, improves recovery from endothelial injury by promoting endothelial cell growth and reduces SMC proliferation (Mendelsohn, 2002). Recent clinical trials with hormone replacement therapy in postmenopausal women have shown that this treatment alone does not reduce the risk of death from cardiovascular disease (Hulley *et al.*, 1998). The area of hormonal benefits in cardiovascular disease requires further research and suggests that correcting other underlying risk factors such as obesity and lifestyle would be more beneficial than hormonal replacement.

Atherosclerosis progresses slowly throughout life and the causal factors exert their effects over a long time. At age 45-54 years, the incidence of coronary heart disease in men is 10 per 1000 and this increases steeply to 30 per 1000 in 75-84 year old men (Franklin *et al.*, 2001; Lakatta & Levy, 2003). Between the ages of 20-90 years, the thickness of the carotid wall increases 2-3 fold (Lakatta & Levy, 2003). One particular view is that the pathophysiology of atherosclerosis interacts with "intrinsic vascular aging" and this is modulated by the typical risk factors such as dyslipidemia, hypertension, hyperglycemia, and cigarette smoking

(Lakatta & Levy, 2003).

Modifiable risk factors for the disease include hypertension, dyslipidemia, type 2 diabetes and cigarette smoking (Shantaram, 1999). Hypertension and coronary heart disease show a positive log-linear relationship with no apparent threshold (MacMahon *et al.*, 1990). Angiotensin II (AII), a product of the renin-angiotensin system, is thought to have a central role in regulating both hypertension and the pathophysiology of macrovascular disease. As well as being a potent vasoconstrictor, AII regulates cell phenotype/growth (Kim *et al.*, 1994), gene expression of other atherogenic factors such as transforming growth factor (TGF)-β1 (Gibbons *et al.*, 1992) and activates multiple intracellular signalling cascades in smooth muscle and endothelial cells (Kim & Iwao, 2000). An overview of early clinical studies with antihypertensive agents such as thiazide diuretics, showed only moderate improvements in death from coronary heart disease when blood pressure was reduced (Collins *et al.*, 1990). Recently, antihypertensive treatment of high risk patients with agents that have improved and direct vascular actions such as the angiotensin converting enzyme (ACE) inhibitor, ramipril, showed a 37% reduction in the risk of death from cardiovascular disease (Heart Outcomes Prevention Evaluation Study Investigators, 2000).

The incidence of CHD correlates directly with the number of cigarettes smoked per day, probably by an activation of the coagulation system. *In vitro* studies have shown that cigarette smoke reduces endothelial prostaglandin I_2 synthesis and increases platelet aggregation (Thompson & Smith, 1989). Additionally, smokers have increased blood fibrinogen levels (Muir, 1992).

Dyslipidemia in subjects susceptible to macrovascular disease show increased LDL, small dense LDL and triglycerides and reduced HDL. High-density lipoprotein is involved in reverse cholesterol transport which involves the removal of cholesterol from the tissues and

subsequent transport to the liver for metabolism and excretion. The increased incidence of macrovascular disease in patients with dyslipidemia is due to excess lipid being trapped in the vessels and the presence of small dense LDL which facilitates deposition and retention.

Cardiovascular disease develops in the setting of diabetes at a rate 3-4 fold that of people without diabetes (Lehto *et al.*, 1997). There are four recognized molecular mechanisms for increased vascular disease in diabetes which all involve over production of reactive oxygen species induced by hyperglycemia; 1) polyol pathway flux, 2) increased advanced glycation end products (AGEs) 3) activation of protein kinase C and 4) increased hexosamine pathway flux (Brownlee, 2001). The latter three mechanisms have been studied in relation to the "response to retention" hypothesis of atherogenesis and are discussed in the next section.

1.4.3   Diabetes factors and the "response-to-retention" hypothesis

Important metabolic and vascular atherogenic factors have been studied in relation to the "response-to-retention" hypothesis. Studies performed *in vitro* show that human vascular smooth muscle cells treated with various AGEs do not alter proteoglycan synthesis (Ballinger & Little, unpublished observations). The basic concept of altered proteoglycan synthesis under high glucose conditions, which involves excess glucose entering the hexosamine pathway, have been unable to conclude whether or not high glucose increases proteoglycan size and thereby the propensity to bind to LDL (Tannock *et al.*, 2002a). The same study attempted to mimic increased extracellular glucose by treating SMCs with exogenous glucosamine but surprisingly, the proteoglycans synthesized in the presence of glucosamine were smaller and showed reduced binding to LDL compared to untreated cells (Tannock *et al.*, 2002a).

Angiotensin II is a potent vasoconstrictor and the receptors for AII may be upregulated in experimental models of diabetes (Sechi *et al.*, 1994) or rat SMCs subjected to high glucose

conditions (Sodhi *et al.*, 2003). Human (Figueroa & Vijayagopal, 2002) and rat (Shimizu-Hirota *et al.*, 2001) smooth muscle cells treated with angiotensin II, show a concentration and time-dependent increase in proteoglycan synthesis and glycosaminoglycan length. These changes in proteoglycans from vascular SMCs treated with angiotensin II are associated with an increased affinity for LDL (Figueroa & Vijayagopal, 2002). The effect of AII treatment of SMCs to increase proteoglycan synthesis could be inhibited by both broad protein kinase C inhibitors and tyrosine kinase inhibitors (Figueroa & Vijayagopal, 2002; Shimizu-Hirota *et al.*, 2001). Direct protein kinase C activation with phorbol myristate acetate in human SMCs, increased proteoglycan synthesis 2.3 fold (Figueroa & Vijayagopal, 2002). Because hyperglycemia may also directly activate protein kinase C, this pathway may be important in amplifying the retention of LDL by stimulating longer glycosaminoglycans, in vessels of subjects with diabetes.

Whether or not the lipid changes (increased triglycerides, small dense LDL and non-esterified fatty acids) in diabetes affect binding to proteoglycans has also been investigated. A recent study has demonstrated somewhat surprisingly, that triglyceride (TG)-rich lipoprotein isolated from people with diabetes do not show increased binding to vascular derived biglycan or the LDL receptor, *in vitro*, compared to lipoprotein from subjects without diabetes (Tannock *et al.*, 2002b). These data are supported by a study which showed that arterial proteoglycans are most reactive with small dense LDL and intermediate-density lipoprotein (IDL), rather than TG-rich very-low-density lipoprotein (Anber *et al.*, 1997).

The catabolism of triglycerides in VLDL and chylomicrons involves lipolysis in the intestine or focal lipolysis in the blood vessels, by endothelial lipoprotein lipase. Triglyceride metabolites, called non-esterified fatty acid (NEFA), are bound to albumin and transported to adipose tissue for storage, a process mediated by insulin. In diabetes, NEFA storage is

inefficient and patients have elevated plasma albumin-bound NEFA (Reaven *et al.*, 1988). *In vitro* studies have shown that human vascular SMC treated with albumin-bound NEFA stimulates the expression of proteoglycan core proteins, versican and decorin (Olsson *et al.*, 1999). Additionally, this study showed that albumin-bound NEFA contributes to increased binding of matrix to LDL (Olsson *et al.*, 1999). Thus, these findings suggest that lipoprotein abnormalities in diabetes may contribute to enhanced lipid retention by proteoglycans in atherogenesis.

The ratio of heparan sulfate (HS) proteoglycans to dermatan sulfate (DS) proteoglycans has been shown to be decreased in the plaques of subjects with diabetes compared to plaques from normal subjects (Wasty *et al.*, 1993). Since HS proteoglycans (PGs) have anti-migratory and anti-proliferative effects, this study concluded that the reduction of HSPGs in diabetes might exacerbate SMC migration and proliferation in these patients. An alternative interpretation of this study could be based on the relative affinity of the proteoglycans to LDL. Proteoglycans with DS GAGs have a higher binding affinity to LDL than the HS proteoglycans (Cardoso & Mourao, 1994; Gigli *et al.*, 1993), and this could lead to more retention of LDL and increased susceptibility to rupture in the diabetic plaque than in the non-diabetic plaque. (See section 1.7.2 for molecular characteristics of glycosaminoglycan chains).

1.4.4 Non-invasive treatment of vascular complications in diabetes

Vascular complications in diabetes are considered in two categories; microvascular disease and macrovascular disease. Microvascular disease (affecting capillaries and small blood vessels) is the most common complication of type 1 diabetes but is also manifest in type 2 diabetes as retinopathy, neuropathy and nephropathy. Macrovascular disease, such as atherosclerosis is the most common cause for mortality and morbidity in type 2 diabetes

patients, but is becoming an important complication in type 1 diabetes (Figure 1-2). Results from the UKPDS indicated that intensive glucose control can reduce microvascular disease but not macrovascular disease in type 2 diabetes (UK Prospective Diabetes Study Group, 1998b).

Multi-factorial modifications to diet, increased physical activity including weight training and decreasing cigarette smoking are the recommended lifestyle changes to prevent cardiovascular disease (Gaede *et al.*, 2003; Hu *et al.*, 2001; Stefanick *et al.*, 1998). Patients with diabetes are also encouraged to take daily low-dose aspirin, an inhibitor of platelet aggregation, as it has been shown to be beneficial for reducing macro- and microvascular complications in patients with diabetes who have another risk factor such as hypertension (Hansson *et al.*, 1998).

Therapies engaged for the treatment of atherosclerosis have been restricted to treating one or more clinically manifest risk factors, namely ACE inhibitors for hypertension, statins for increased LDL cholesterol, sulfonylureas and glitazones (i.e. PPAR-γ ligands) for hyperglycemia of diabetes and fibric acid derivatives (i.e. PPAR-α ligands) for hypertriglyceridemia and low HDL levels. It is has been shown that some of these therapies, while targeting the specific risk factor, also have "pleiotropic" actions including anti-atherogenic effects on the vasculature. For example it has been shown that statins reduce vascular smooth muscle cell migration and proliferation independent of their cholesterol lowering effect (Bellosta *et al.*, 1998). In LDL receptor knockout mouse studies, a PPAR-γ ligand, troglitazone was shown to reduce atherosclerosis by preventing lipid deposition (Law *et al.*, 1996). Additionally, the DAIS showed a 40% reduction in coronary narrowing with 3 years of fenofibrate treatment in a group of patients with type 2 diabetes (Diabetes Atherosclerosis Intervention Study Investigators, 2001). The UKPDS showed that aggressive control of blood pressure with an ACE inhibitor is more beneficial for the risk reduction in

cardiovascular disease than by intensive glucose control although the blood pressure control exceeded that attained for glucose control (UK Prospective Diabetes Study Group, 1998b). Hence, it is well accepted that there are beneficial effects of cardiovascular risk factor reduction therapies on atherosclerosis, independent of their primary action.

1.4.5   Invasive treatment of macrovascular complications in diabetes

Invasive treatment of macrovascular disease aims to reopen partially occluded or nearly occluded vessels. Percutaneous transluminal coronary angioplasty (PTCA) involves the insertion of a balloon catheter which is inflated at the obstructed site to stretch the arterial wall and squash the plaque (Figure 1-8A). The PTCA procedure has been successful in ameliorating atherosclerosis but unfortunately in patients with the added burden of diabetes this form of treatment has reduced applicability due to increased rates of restenosis and vascular smooth muscle cell hyperplasia (Kornowski *et al.*, 1997). An alternative method to re-vascularization is coronary artery bypass grafting (CABG) which involves the transplant of healthy vessels from other beds such as the internal mammary artery (IMA), radial artery (RA) or saphenous vein (SV), to bypass the site of occlusion (Figure 1-8B). The order of success of the three vessels in transplant in terms of susceptibility to developing atherosclerosis has been in the order of IMA=RA>SV (Buxton *et al.*, 1997; Grondin *et al.*, 1984; Iaffaldano *et al.*, 1995). The grafted vessels may eventually develop atherosclerosis, regardless of their original location, within 5-12 years (Lytle *et al.*, 1985; Mautner *et al.*, 1992). The limited success of invasive treatments in diabetes may be a result of the incomplete understanding of the biological mechanisms which control and contribute to the development of atherosclerosis in these patients and the continuation of the underlying disease as an ongoing driver of atherosclerosis.

A

Balloon | Atherosclerotic | Narrowed lumen | Coronary
plaque | of artery | artery

Balloon catheter with uninflated balloon is threaded
to obstructed area in artery

When balloon is inflated, it stretches arterial wall
and squashes atherosclerotic plaque

After lumen is widened, balloon is deflated and catheter
is withdrawn

B

Ascending
aorta

Grafted
vessel

Obstruction

Figure 1-8

Invasive procedures for the amelioration of atherosclerotic lesions. (A) Percutaneous transluminal coronary angioplasty (PTCA) and (B) coronary artery bypass grafting. From (Tortora & Grabowski, 1996).

1.5 The extracellular matrix of normal and atherosclerotic vessels

1.5.1 Distribution of glycosaminoglycans in large vessels

Proteoglycans along with collagen and elastic fibres fill the extracellular space in the vessel wall (Wight, 1980). The bulk of the vascular glycosaminoglycans are synthesized by smooth muscle cells, endothelial cells and fibroblasts and are concentrated in the extracellular matrix of the intima and adventitia layers of the blood vessel (Wight, 1999). Proteoglycans consist of one or more glycosaminoglycan chain(s) covalently linked to a core protein (see section 1.7) and the vascular proteoglycans include perlecan, versican, biglycan, decorin and osteoglycin. The non-protein associated glycosaminoglycan (GAG), hyaluronan, is also present in blood vessels.

Localization of proteoglycans in human non-atherosclerotic coronary arteries, most commonly by immunohistochemical identification of core proteins, has shown that there is a large amount of versican in the intima of normal vessels, with scattered deposits of this proteoglycan in the media and adventitia layers (O'Brien *et al.*, 1998). In contrast, biglycan is found in the adventitia close to the external elastic lamina with minimal deposits in the media and intima layers (O'Brien *et al.*, 1998). In normal, non-human primate vessels, perlecan and hyaluronan are prominent in the endothelial basement membrane and intima, respectively while versican, biglycan and decorin show scanty expression in this area (Evanko *et al.*, 1998; Wight, 1999). In the adventitia layer, decorin and hyaluronan are expressed with minimal deposits of perlecan, versican or biglycan in this area (Evanko *et al.*, 1998). Osteoglycin, a keratan sulfate proteoglycan predominantly found in the cornea of the eye, has been reported to be expressed in the media and adventitia layers of normal adult rat carotid artery (Funderburgh *et al.*, 1997; Shanahan *et al.*, 1997), human coronary artery (Funderburgh *et al.*, 1997; Shanahan *et al.*, 1997) and rabbit aorta (Fernandez *et al.*, 2003).

1.5.2 Distribution of glycosaminoglycans in blood vessels with atherosclerosis

The first description of an association of proteoglycans with atherosclerosis susceptibility was reported in 1965 (Mancini *et al.*, 1965). This report described vessels from atherosclerotic-susceptible species (chickens, pigeons, rabbits) to contain a greater concentration of proteoglycans than vessels from atherosclerosis-resistant species (rats, mice, hamsters) (Mancini *et al.*, 1965). Subsequent studies have shown that the amount of vascular glycosaminoglycans in the atherosclerosis-susceptible pigeon (White Carneau; WC) is greater than the amount of GAGs in the vessels of the atherosclerosis-resistant pigeon (Show Racer; SR) (Curwen & Smith, 1977; Edwards & Wagner, 1988). Additionally, GAGs from the thoracic aorta of the WC pigeon contain more DS than GAGs from the aortae of the SR pigeon (Curwen & Smith, 1977). Immunohistochemical localization of proteoglycan core proteins versican and biglycan in human coronary atherosclerotic plaques showed that there is an increase in biglycan and versican deposits compared to normal vessels, with biglycan accumulation in areas without versican (O'Brien *et al.*, 1998). Non-human primate vessels have been used to show that the distribution of proteoglycans is dynamic during the atherogenic process (Evanko *et al.*, 1998). From this report, intermediate lesions showed an intense staining of versican, biglycan, decorin and hyaluronan in the intimal region with minimal staining of perlecan in this area (Evanko *et al.*, 1998). The endothelial basement membrane and the media layer showed similar proteoglycan and GAG distributions as the non-atherosclerotic vessel (Evanko *et al.*, 1998). In advanced atherosclerosis, proteoglycan localization was associated with the structural features of the lesion. For example, perlecan was prominent around the margins of the plaque core in SMCs, while versican was found mostly in the fibrous cap. Biglycan was widely distributed but mostly in the fibrous cap and decorin was localized to macrophage rich areas of the plaque core. Hyaluronan was associated

with versican staining in the fibrous cap however, it was also prominently deposited within the plaque core (Evanko *et al.*, 1998). The reduced HSPGs and increased DSPGs pattern of glycosaminoglycan expression in the atherosclerotic lesion, is similar and more pronounced in atherosclerotic plaques from patients with diabetes (Wasty *et al.*, 1993).

Other extracellular matrix components, such as collagen, increase with lesion severity and can be correlated with specific proteoglycans which bind to collagen, i.e. decorin (Wight, 1989). Decorin interacts with type I collagen via its core protein to regulate fibrillogenesis (Vogel *et al.*, 1984). The expression of osteoglycin is reduced in the media of the atherosclerotic vessel compared to the normal vessel and seems be associated with non-proliferative SMCs (Shanahan *et al.*, 1997).

1.6   Mechanisms of atherogenesis and regulation by glycosaminoglycans

Proteoglycans, such as biglycan and decorin, have been described and characterized in vascular cells (Jarvelainen *et al.*, 1991). Proteoglycans, which are either secreted from the cell or associated with the cell surface, form a small component of the blood vessel mass although they have a number of physical and biological roles in the normal blood vessel. Importantly proteoglycans modulate the proliferation and migration of SMCs, the retention of LDL and the formation of foam cells, the basis of the 'response to injury' and 'response to retention' hypotheses of atherogenesis.

1.6.1   Glycosaminoglycans and the proliferation and migration of vascular cells

The smooth muscle cell is known to have a central role in the development and progression of atherosclerosis (Campbell *et al.*, 1987; Chamley-Campbell *et al.*, 1979). Injury to the endothelium is thought to initiate the migration and proliferation of SMCs from the media, to the sub-endothelial area, forming a larger intima (Ross & Glomset, 1976a, 1976b). Endothelial

damage can arise from the continual mechanical stress at atherosclerotic prone sites of the arterial tree. The SMCs which migrate and proliferate to form the intima take on a synthetic phenotype, synthesizing connective tissue and growth factors and can stimulate themselves as well as neighbouring cells to migrate, proliferate and synthesize extracellular matrix (Ross, 1993).

Endothelial cell injury precedes an increase in the amount of chondroitin sulfate (CS)PG and LDL in the intima. *In vitro* studies have shown that proliferating SMCs produce a greater amount of CSPG than quiescent cultures (Camejo *et al.*, 1993). Heparan sulfate proteoglycans such as perlecan, inhibit the proliferation of SMCs (Weiser *et al.*, 1997), however in the atherosclerotic vessel the amount of perlecan is reduced which may permit cellular infiltration and proliferation (Evanko *et al.*, 1998). Additionally, HSPGs are the major type ($\approx$80%) of proteoglycan synthesized by endothelial cells, yet after endothelial cell wounding these cells produce less HSPGs and more CS/DS proteoglycans (Kinsella & Wight, 1986).

Hyaluronan is associated with areas of the atherosclerotic lesion where there is active smooth muscle cell proliferation and migration (Riessen *et al.*, 1996). *In vitro* studies have shown that the proliferating SMC has a pericellular coat of hyaluronan which is bound to CD44 receptors and Receptor for HyAluronan-Mediated Motility (RHAMM) on the smooth muscle cell surface (Evanko *et al.*, 1999; Savani *et al.*, 1995).

Heparin, a non-protein associated GAG is a potent inhibitor of smooth muscle cell proliferation and migration (Clowes & Karnowsky, 1977). In contrast to SMCs, the growth of endothelial cells is not inhibited by heparin. The presence of heparin, and other sulfated GAGs stimulates the proliferation and migration of endothelial cells (Klein-Soyer *et al.*, 1989; Rosenbaum *et al.*, 1986; Thornton *et al.*, 1983). Similar to heparin, hyaluronan oligosaccharides inhibit the migration and proliferation of smooth muscle cells but stimulate

these processes in endothelial cells (Savani *et al.*, 1995).

In vitro studies using cultured rat vascular SMCs have shown that osteoglycin is down-regulated in the presence of serum and atherogenic growth factors, such as platelet derived growth factor (PDGF), TGF-β1 and the hormone, angiotensin II (Shanahan *et al.*, 1997). The expression of osteoglycin is correlated with non-proliferative SMCs suggesting that this proteoglycan is not required for vascular SMC proliferation and migration in the atherogenic process.

1.6.2 Retention of low-density lipoprotein by the extracellular matrix

Glycosaminoglycans comprise only $\approx 0.01\%$ of the blood vessel wet weight mass (Vijayagopal *et al.*, 1985) however, they play an important role in maintaining the turgidity of the vessel. Proteoglycans are responsible for a number of cellular processes within the arterial wall (Wight, 1989). Of interest, is the influence of proteoglycans in lipid binding, retention and deposition, which is a process thought to initiate atherosclerosis. The interaction of proteoglycans with lipoproteins is mediated by positively charged segments of apolipoprotein-B100 (ApoB100), the apolipoprotein associated with LDL (Wiklund *et al.*, 1996). The binding between proteoglycans and LDL is specific because ApoB100 has been shown to contain eight GAG binding regions characterized by clusters of basic amino acids (Camejo *et al.*, 1988; Hirose *et al.*, 1987; Weisgraber & Rall, 1987). Further studies have shown that a single point mutation in the ApoB100 eliminates proteoglycan binding and binding to the LDL receptor (Boren *et al.*, 1998). It has been suggested that the protein fold (Figure 1-9) of the carboxyl-terminal domain of ApoB100 hides another GAG binding site, which is exposed in ApoB80 and ApoB48 (80% and 48% of ApoB, respectively) (Flood *et al.*, 2002).

The length of the proteoglycan GAG chains correlates with an increased ability to retain lipoprotein (Camejo *et al.*, 1993; Chang *et al.*, 2000; Little *et al.*, 2002; Schonherr *et al.*, 1993). In atherosclerosis, the combination of atherogenic growth factors and the change of the SMC to a synthetic phenotype in the intima results in an increased rate of proteoglycan synthesis which involves lengthening of the GAGs and in experimental models of atherosclerosis, a modification in the sulfation pattern of the GAGs from 4-sulfate to 6-sulfate (Edwards & Wagner, 1988). As previously mentioned, the work of Boren and colleagues (Skalen *et al.*, 2002) has demonstrated and supported the "response-to-retention" hypothesis using a mouse model of atherogenesis. Additionally, the proteoglycans from the atherosclerosis model in the pigeon had longer glycosaminoglycan chains and an alteration in the sulfation pattern compared to the atherosclerosis resistant model (Edwards & Wagner, 1988). In an injury model of atherosclerosis, chondroitin sulfate GAGs were shown to colocalize with apoB (Galis *et al.*, 1993). Immunohistochemical staining of human plaques have shown that biglycan was colocalized with apoE and suggested that lipoproteins other than LDL, such as VLDL remnants and a subclass of HDL containing apoE are also retained by biglycan (O'Brien *et al.*, 1998).

The structural and compositional changes of proteoglycans within atherosclerotic lesions are likely to increase the propensity to bind to LDL. For example, comparative studies have shown that CSPGs isolated from atherosclerotic lesions are different from proteoglycans within the normal blood vessel because the length of the GAG chains is increased despite a reduction in the number of GAGs on the core protein and the GAGs have more sulfate groups at the carbon-6 position (Wagner *et al.*, 1986). Multiple *in vitro* studies have shown that an increase in GAG length is correlated with increased binding to LDL. This has been demonstrated following stimulation of vascular SMCs with TGF-β1 (Little *et al.*, 2002), free

fatty acids (Olsson *et al.*, 1999), oxidised LDL (Chang *et al.*, 2000), angiotensin II (Figueroa & Vijayagopal, 2002) and proliferating SMCs (Camejo *et al.*, 1993).
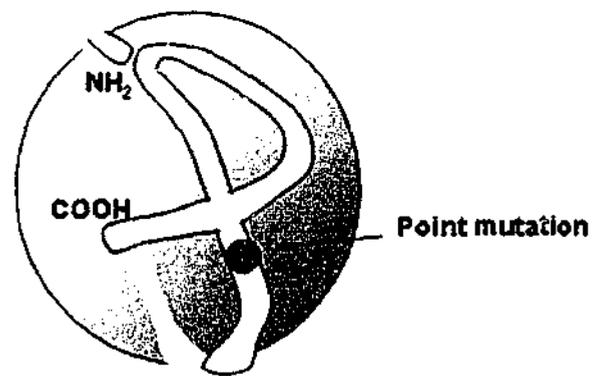
Figure 1-9

Representation of apolipoprotein (Apo)B100 associated with low-density lipoprotein (LDL). Starting with the amino-terminal, ApoB100 wraps around the LDL particle however, the carboxyl-terminal folds over itself, covering a glycosaminoglycan binding site. A single point mutation in the ApoB100 prevents binding to proteoglycans. Adapted from (Flood *et al.*, 2002).

Whether the proteoglycans have CS, DS or HS GAGs also determines the affinity for LDL. The iduronic acid content and 4-sulfation features of DS have been reported to give structural stability to the PG-LDL complex (Gigli *et al.*, 1993). Two studies have shown that DS bind with almost one order of magnitude higher affinity to LDL than CS GAGs at equivalent levels of sulfation (Gigli *et al.*, 1993) or charge density (Iverius, 1972). In preparations of proteoglycans isolated from human arteries, those that had a high affinity for LDL contained a higher proportion of DS than the proteoglycans that had a low affinity for LDL (Cardoso & Mourao, 1994). Although heparan sulfate proteoglycans may contain a higher amount of iduronic acid than CS/DS proteoglycans, they show lower affinity for LDL than CS/DS proteoglycans (Vijayagopal *et al.*, 1981) and this may be due to low sulfation levels.

There are mixed reports relating to the position of the sulfate group at the C4 or C6 position on the disaccharides which make up the GAGs and whether or not one position or the other results in greater affinity to LDL. While proteoglycans isolated from atherosclerotic lesions show mostly 6-sulfation in humans and animals (Wight, 1980) *in vitro* data suggests that 4-sulfation provides structural stability of the proteoglycan-LDL interaction (Gigli *et al.*, 1993).

1.6.3 Glycosaminoglycans associated with macrophages and foam cell formation

Circulating monocytes detect accumulated lipid in the matrix and surrounding cells. Monocytes adhere to the endothelial layer by specific adhesive glycoproteins on the surface of the endothelial cells, traverse the endothelium and enter the intima where they differentiate into macrophages and attempt to digest the retained lipid. Increased lipid overwhelms macrophage LDL degradation processes and the cholesterol remains in the macrophages. The lipid-laden macrophages are deposited as foam cells in the sub-endothelial space, which are

macroscopically visualised as a fatty streak. With continual lipid deposition and macrophage recruitment, the fatty streak progresses to an intermediate lesion. The lesion is capped with fibrotic and calcified tissue, while the centre of the lesion may undergo necrosis and this is known as a complex lesion. The plaque may be stable or if continual stress occurs may rupture and cause occlusion of the affected and surrounding vessels.

Modifications of LDL by arterial proteoglycans may contribute to the formation of foam cells. Uptake of LDL bound to chondroitin 6-sulfate proteoglycans into human monocyte derived macrophages is enhanced 2-4 times more than unbound LDL and increases the conversion to foam cells (Hurt & Camejo, 1987; Hurt-Camejo *et al.*, 1990). Smooth muscle cells are also capable of internalising LDL-proteoglycan complexes (Ismail *et al.*, 1994).

The increase in hyaluronan in atherosclerosis may exacerbate the recruitment of monocytes to the lesion. For example, in the inflammatory response to bowel disease, mononuclear leukocytes (U937 cells) adhere to mucosal smooth muscle cells by interacting with hyaluronan (de La Motte *et al.*, 1999). The interaction with hyaluronan occurs partly because the leukocytes express CD44, a cell surface hyaluronan binding protein. It is hypothesized that monocytes would interact with hyaluronan in the vessel wall during the atherogenic process by a similar mechanism as monocytes also express CD44 (Culty *et al.*, 1994).

1.7 Biosynthesis of vascular glycosaminoglycans

Proteoglycans are widely expressed throughout mammalian tissues however the type and amount of various proteoglycans differs between tissues. For example aggrecan, a CS/keratan sulfate (KS) proteoglycan, is the major proteoglycan found in cartilage, while mesangial cells synthesize predominantly HSPGs. Additionally different cells within the same tissue will synthesize variable amounts and types of proteoglycans. For instance, smooth muscle cells

synthesize mostly CS/DS proteoglycans and a small amount of HSPGs while endothelial cells secrete more HSPGs over CS/DS PGs (Kinsella & Wight, 1986). The type and nature of proteoglycans may also alter under the influence of various agents in a particular cell type such as PDGF treatment of vascular SMCs which increases the expression of versican and biglycan core proteins and increases the length of the GAG chains (Schonherr *et al.*, 1993; Schonherr *et al.*, 1991).

Proteoglycans/glycosaminoglycans can be located in the extracellular matrix (e.g. versican, biglycan and decorin), associated with specialized structures of the extracellular matrix such as the basement membrane (e.g. perlecan), part of the plasma membrane (e.g. syndecans) or within intracellular structures such as secretory storage granules (e.g. heparin). Proteoglycans are composed of a protein core with one or more covalently linked GAG side chains (Wight, 1989). The most abundant proteoglycans secreted by smooth muscle cells are the CSPG, versican which has between ten and thirty GAG chains, the CS/DS proteoglycans, biglycan which has two GAG chains and decorin which has one GAG chain (Wight, 1995) and a HS/CS proteoglycan, called perlecan which has between two and three GAG chains. Hyaluronan is a non-sulfated, non-protein-associated glycosaminoglycan and is also synthesized by SMCs. For the purpose of this thesis, the biosynthesis of the vascular glycosaminoglycans will be described.

1.7.1 Proteoglycan core protein

The deoxyribonucleic acid (DNA) encoding proteoglycan core proteins, undergoes transcription to messenger ribonucleic acid (mRNA), in the cell nucleus followed by mRNA translation to protein in the rough endoplasmic reticulum. The core protein contains specific amino acid sequences that determine the post-translational modifications, such as the Ser-Gly

dipeptide for covalent glycosaminoglycan attachment (Zhang *et al.*, 1995).

1.7.1.1   *Perlecan core protein*

Perlecan was isolated from vascular smooth muscle cells in 1991 (Noonan *et al.*, 1991). Perlecan has a large core protein (400-467 kDa) with five domains, each of which has been shown to be involved in various cellular functions (Figure 1-10A). The N-terminal domain (Domain 1) of perlecan contains three GAG attachment sites, which are predominantly heparan sulfate chains but may also be chondroitin sulfate chains (Costell *et al.*, 1997; Dolan *et al.*, 1997; Groffen *et al.*, 1996; Kokenyesi & Silbert, 1995). Domain II of perlecan resembles the ligand-binding portion of the LDL receptor however, rather than bind to LDL this domain is thought to facilitate the interaction with lipid bilayers for the arrangement of basement membranes (Iozzo & Danielson, 1999; Noonan & Hassell, 1993). Domain III shows similarities to parts of laminin-1 (Timpl & Brown, 1994), and may act with other regions in the protein core to promote integrin-mediated cell attachment (Iozzo, 1998). Domain IV contains 21 immunoglobulin (Ig)-like repeats and resembles a string of pearls hence, the name perlecan (Figure 1-10A). Domain IV may be involved in dimerization or self-association, while domain V is thought to have a role in basement membrane formation (Iozzo, 1998; Yurchenco *et al.*, 1987).

1.7.1.2   *Versican core protein*

Versican core protein is classified as a hyalectin because two out of its three domains specifically bind to different saccharides (Figure 1-10B). Domain 1 (N-terminal domain) of versican contains a hyaluronan binding region, domain II contains two sub-domains, GAG-α and GAG-β, which are GAG attachment sites capable of linking thirty chains. Domain III

(C-terminal domain) is a lectin binding domain which is involved in binding to simple carbohydrates (lectins) in a calcium-dependent manner (Iozzo & Murdoch, 1996; Kishore *et al.*, 1997). The versican core protein has a variable molecular weight (265-370 kDa) depending on the splice variant expressed. There are four possible splice variants of versican, V0 which contains GAG-α and GAG-β, V1 which contains only the GAG-β region, V2 which contains only the GAG-α region and V3 which does not contain a GAG attachment region. In vascular smooth muscle cells the most common variants expressed are V0 and V1 (Dours-Zimmermann & Zimmermann, 1994). In the vessel wall immunohistochemical staining of versican core protein is closely associated with hyaluronan staining and it is thought that versican and hyaluronan form macromolecular aggregates which attract water molecules (Evanko *et al.*, 1998).

Figure 1-10

Structural features of proteoglycan core proteins. A) Structure of perlecan core protein, Ig, immunoglobulin-like repeat, B) structure of versican core protein, C) structure of decorin core protein, LRR, leucine-rich repeat, D) osteoglycin/mimecan core protein. Adapted from (Funderburgh *et al.*, 1997; Iozzo, 1998).

1.7.1.3 Biglycan/Decorin core proteins

Biglycan and decorin are classified are small leucine-rich proteoglycans because they have similar, small core protein molecular weights (43 kDa and 40 kDa, respectively) and leucine-rich repeats within domain III. Because of the structural similarities between biglycan and decorin core proteins, only the decorin core protein has been illustrated (Figure 1-10C). Domain I contains a signal peptide and a propeptide which has been suggested to be a recognition signal for xylosyltransferase (Sawhney *et al.*, 1991), the first enzyme involved in GAG synthesis. Domain II contains the GAG attachment site for one (decorin) or two (biglycan) chondroitin sulfate/dermatan sulfate chains. Domain III contains the leucine-rich repeats and domain IV contains a large loop with two cysteine residues (Fisher *et al.*, 1989; Krusius & Ruoslahti, 1986; Sawhney *et al.*, 1991; Yanagishita, 1993).

As well as having GAG attachments, proteins such as growth factors can bind to the leucine-rich repeat of biglycan/decorin core protein. Molecular modelling of decorin demonstrates a horse-shoe structure (Figure 1-11) (Iozzo, 1998; Yamaguchi *et al.*, 1990) where the leucine-rich repeat acts as a reservoir for growth factors, such as TGF-β1 (Iozzo, 1998; Ruoslahti & Yamaguchi, 1991; Schlessinger *et al.*, 1995). Decorin core protein also binds to specific regions of collagen fibrils to regulate fibrillogenesis (Vogel *et al.*, 1984).
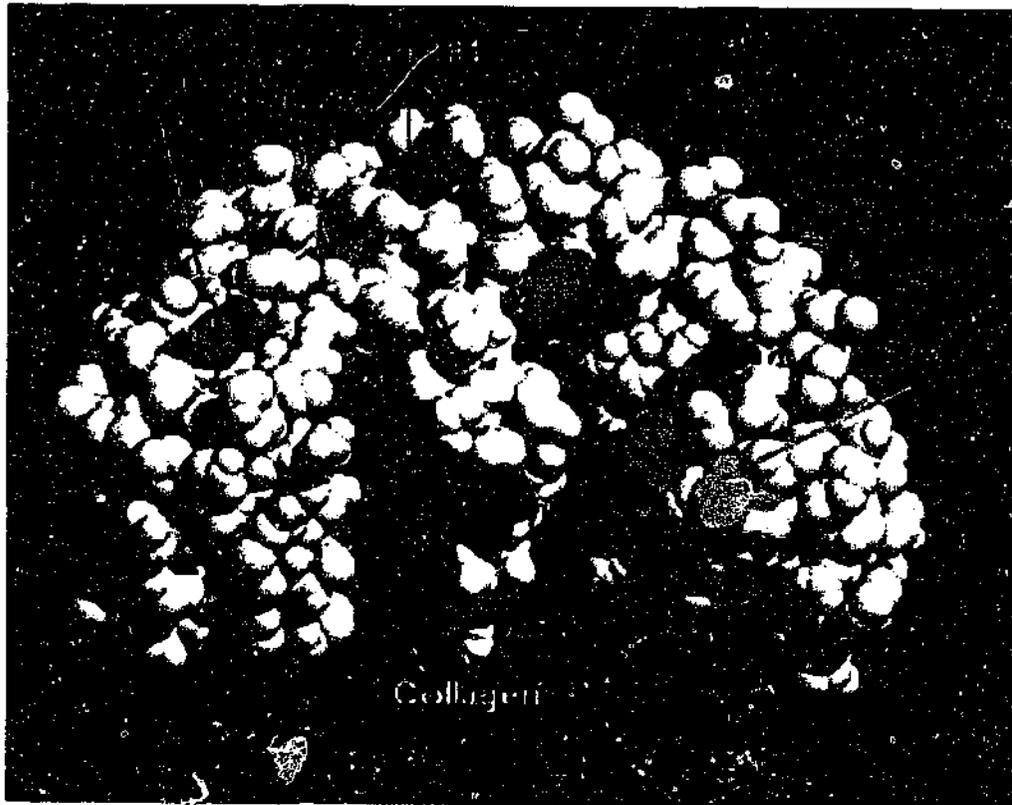
Figure 1-11

Molecular model of decorin core protein. The leucine-rich repeats give the protein a "horse-shoe" appearance that physically interacts with proteins such as collagen (yellow/green). The serine residue (green) is the attachment site for a chondroitin sulfate/dermatan sulfate glycosaminoglycan chain. From (Iozzo, 1999).

1.7.1.4 Osteoglycin/mimecan core protein

Like biglycan and decorin, osteoglycin/mimecan is part of the small leucine rich family of proteoglycan core proteins. Osteoglycin/mimecan core protein has a molecular weight of ≈36 kDa and both the protein and mRNA are expressed in the human aorta (Funderburgh *et al.*, 1997). Mimecan core protein has one keratan sulfate GAG attachment site most probably located near the fifth region of leucine-rich repeats (Figure 1-10D) (Dunlevy *et al.*, 1998).

1.7.1.5 Regulation of core protein synthesis

Darglitazone, a PPAR-γ ligand, reduces decorin expression in human arterial SMCs (Olsson *et al.*, 1999). A recent report has shown that the treatment of hepatocytes with natural and synthetic PPAR-α ligands, reduces versican mRNA expression however, it was stated that the promoter region of versican does not contain a PPAR response element (Olsson *et al.*, 1999). The promoter regions of perlecan, biglycan, (Ungefroren & Krull, 1996), decorin (Iozzo, 1998; Ungefroren & Krull, 1996) and osteoglycin/mimecan (Tasheva, 2002) but not versican contain NF-κB recognition sites and decorin also has an AP-1 site (Table 1-3). It remains to be determined whether or not the AP-1 and NF-κB sites within the promoter regions of the proteoglycan core proteins may be a potential site of transrepression by the PPAR-α ligands (Figure 1-4).

The current understanding of growth factor regulation of core protein expression in vascular cells is that TGF-β1 increases perlecan (Kaji *et al.*, 2000), versican and biglycan core protein expression with no effect on decorin (Schonherr *et al.*, 1993). Vascular SMCs treated with PDGF stimulates versican core protein expression with no effect on biglycan or decorin expression (Schonherr *et al.*, 1993). In other studies, versican and decorin expression is induced by non-esterified free fatty acids (Olsson *et al.*, 1999) and 72 h exposure to oxidized

LDL increases biglycan mRNA (Chang *et al.*, 2000). Growth stimulated vascular SMCs show increased expression of perlecan, versican and biglycan and decreased expression of decorin (Tao *et al.*, 1997).

Core protein	Promoter region sites	
	AP-1	NF-κB
Perlecan	No	Yes
Versican	No	No
Biglycan	No	Yes
Decorin	Yes	Yes
Osteoglycin/mimecan	No	Yes

Table 1-3

The presence and absence of AP-1 and NF-κB sites in the promoter regions of vascular proteoglycans. PPARs induce transrepression of AP-1 and NF-κB regulated genes and may influence the promoter regions of perlecan, biglycan, decorin and mimecan (but not versican) core proteins which may contain one or both of these regions. Adapted from (Iozzo, 1998; Tasheva, 2002; Ungefroren & Krull, 1996).

1.7.2 Glycosaminoglycan chains

Each proteoglycan has a core protein to which one or more glycosaminoglycan chains are covalently attached. The core protein is transported through the Golgi Complex, where GAG elongation occurs (Figure 1-12). The basic construction of GAGs is disaccharide repeats, one monosaccharide is an amino sugar with N-acetyl-glucosamine (GlcNAc) or N-acetyl-galactosamine (GalNAc) and the other unit is an uronic acid, glucuronic acid (GlcA) or iduronic acid (IdoA) (Jackson et $al.$, 1991; Prydz & Dalen, 2000). There are six different groups of GAGs, chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S), dermatan sulfate, heparin/heparan sulfate, keratan sulfate and hyaluronan. Because there is much microheterogeneity in the GAGs, it is hard to discriminate between C6S, C4S and DS so they are combined into one group as chondroitin/dermatan sulfates. With the exception of hyaluronan and heparin, the GAG chains are covalently attached by an O-glycosidic linkage to a serine/threonine residue on a CS/DS/HS core protein or N-linked to an asparagine residue on a KS core protein (Jackson et $al.$, 1991; Schwartz, 2000). The process of linkage region attachment occurs in both the endoplasmic reticulum (ER) and the cis-Golgi of the cell (Figure 1-12) (Kearns et $al.$, 1993; Vertel et $al.$, 1993). The initial hexose of the linkage region is xyloside, followed by galactose (Gal), galactose and GlcA (Figure 1-13) catalyzed by the sequential actions of uridine diphosphate (UDP)-xylosyl transferase, UDP-Gal transferase I, UDP-Gal transferase II and UDP-GlcA transferase I, respectively (Figure 1-12) (Kearns et $al.$, 1993; Vertel et $al.$, 1993).

Sugars such as glucosamine in the cytosol are converted to nucleotide sugar precursors (uridine diphosphate (UDP)-sugars) and together with sulfate, are transported into the endoplasmic reticulum and Golgi lumen by specialized transporters (Hirschberg & Snider, 1987; Mandon et $al.$, 1994). Phosphoadenosylphosphate (PAPS) is the sulfate donor to

sulfotransferases and naturally occurring sulfated cellular products (Prydz & Dalen, 2000; Schwartz, 2000; Yanagishita *et al.*, 1989).

1.7.2.1 Chondroitin sulfate/dermatan sulfate GAGs

Chondroitin sulfate GAGs are composed of glucuronic acid and *N*-acetylgalactosamine (Figure 1-14A). Elongation of CS chains occurs through the alternate action of GalNAc transferase I (Rohrmann *et al.*, 1985) and GlcA transferase II (Prydz & Dalen, 2000; Sugumaran *et al.*, 1997). Dermatan sulfate GAGs arise from the epimerization of the glucuronic acid (in CS chains) to iduronic acid by a C5 uronosyl epimerase (Malmstrom & Fransson, 1975) (Figure 1-14A). The use of brefeldin which destroys the *trans*-Golgi network, in various cell cultures has assisted in the localization of the enzymes required for the synthesis of CS/DS GAGs in the *trans*-Golgi (Calabro & Hascall, 1994; Fransson *et al.*, 1992; Spiro *et al.*, 1991; Sugumaran *et al.*, 1992; Uhlin-Hansen & Yanagishita, 1993). Epimerization tends to occur in segments of the chain rather than conversion of the whole CS chain to DS. Epimerization of CS to DS is closely associated with 4-sulfation (Malmstrom & Fransson, 1975). The GalNAc within CS/DS GAGs are sulfated by 6-*O*-sulfotransferase (Uchimura *et al.*, 1998) and 4-*O*-sulfotransferase (Yamauchi *et al.*, 1999) and occasionally the IdoA on DS GAGs is sulfated by the 2-*O*-sulfotransferase (Figure 1-14A) (Kobayashi *et al.*, 1999).
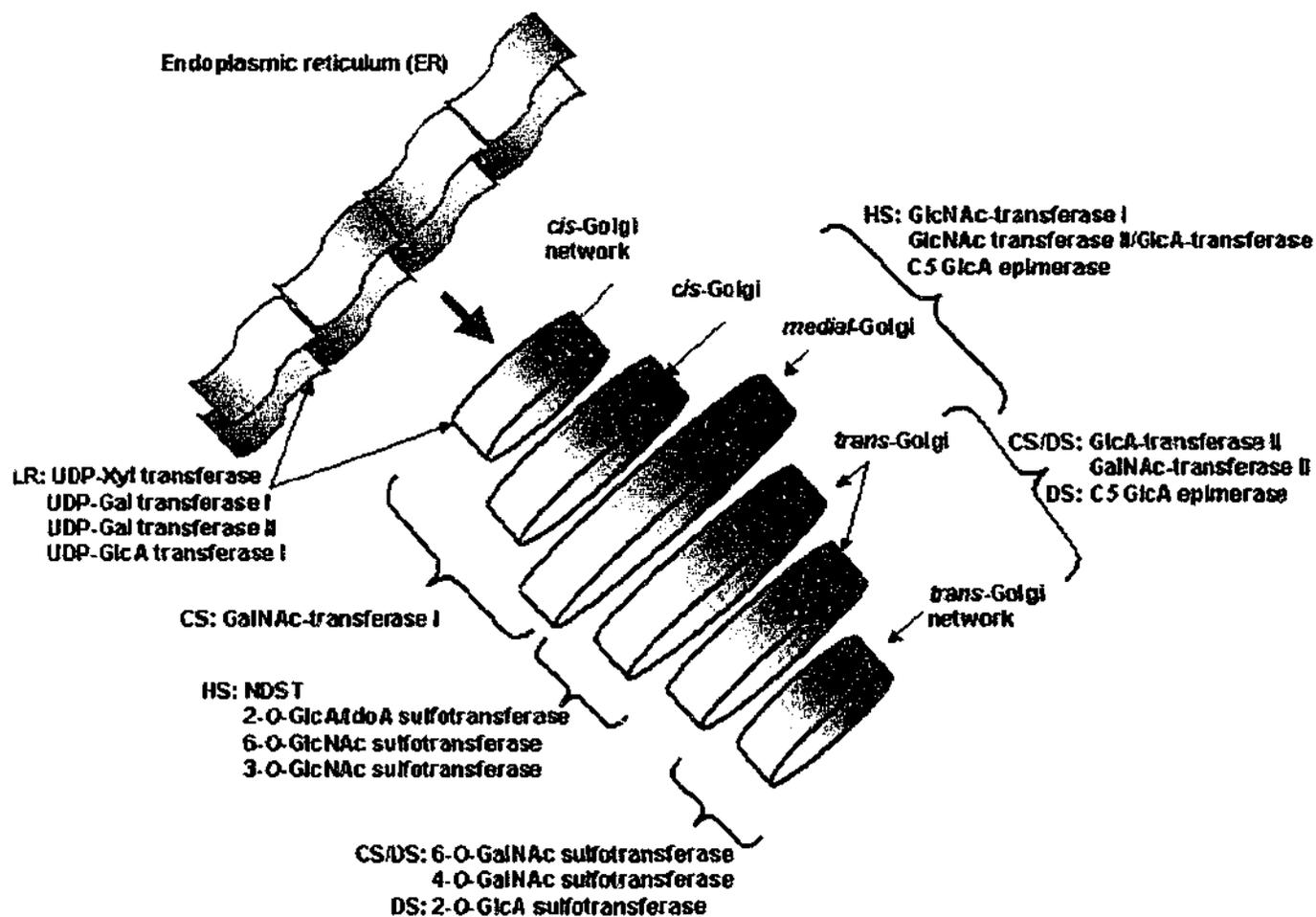
Figure 1-12

The localization of enzymes required for synthesis of chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) GAGs, in the cellular endoplasmic reticulum and the Golgi apparatus. LR - linkage region, Xyl - xylose, Gal - galactose, GlcA - glucuronic acid, GlcNAc - *N*-Acetylglucosamine, GalNAc - *N*-Acetylgalactosamine, NDST - GlcNAc *N*-deacetylase/*N*-sulfotransferase. Red arrow indicates direction of core protein through the Golgi. Adapted from (Prydz & Dalen, 2000; Tortora & Grabowski, 1996).
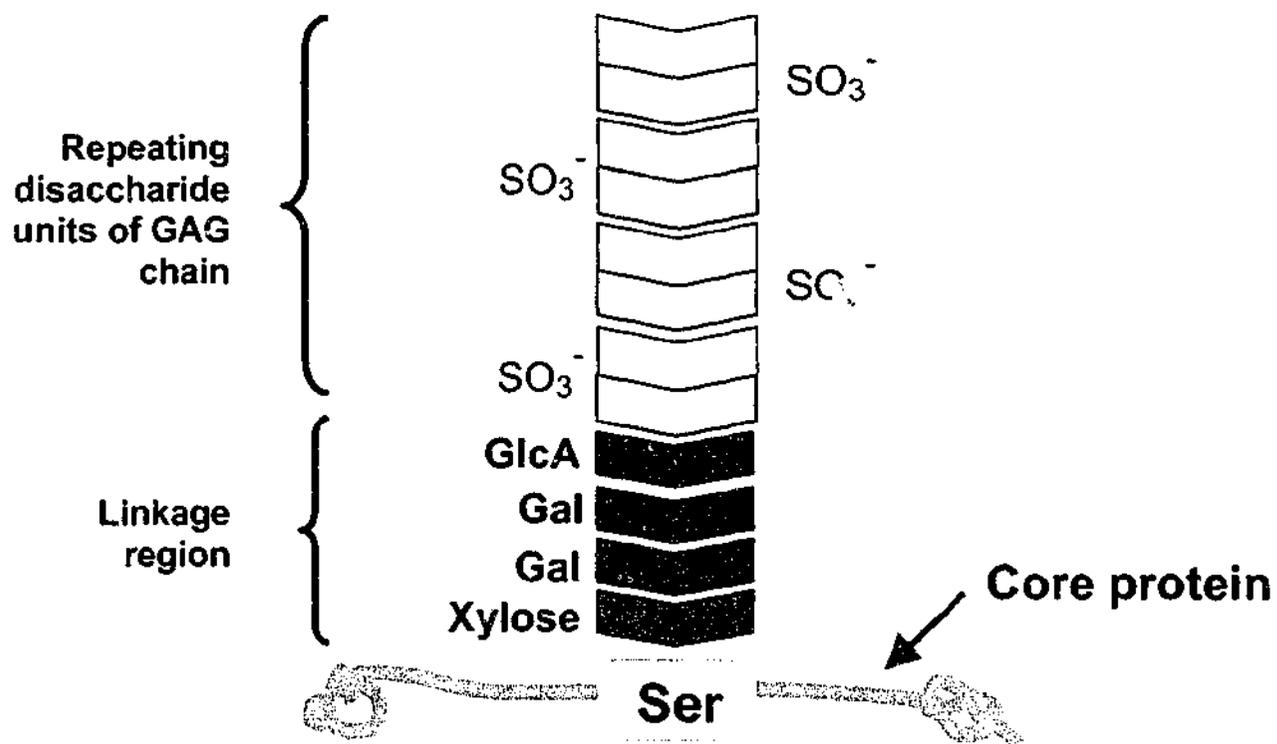
Figure 1-13

Stylized structure of a proteoglycan - each glycosaminoglycan chain is attached to a core protein by a common tetrameric linkage. Xylose is the initial pentose linked to the serine on the core protein followed by the sequential addition of two galactose (Gal) units and glucuronic acid (GlcA). With the exception of hyaluronan and heparin, the GAG chains are covalently attached by an *O*-glycosidic linkage to a serine (ser)/threonine on a CS/DS/HS core protein or *N*-linked to asparagine on a KS core protein (Jackson *et al.*, 1991; Schwartz, 2000).

1.7.2.2   *Heparin/heparan sulfate GAGs*

The disaccharide repeat for heparin/heparan sulfate GAGs is glucuronic acid and *N*-acetylglucosamine joined by $\beta(1\rightarrow4)$ linkages only, unlike all other GAGs which have alternating $\beta(1\rightarrow4)$, $\beta(1\rightarrow3)$ linkages (Prydz & Dalen, 2000; Wight *et al.*, 1991) (Figure 1-14A and 1-14B). These GAGs are extensively modified by sulfotransferases and an epimerase. Elongation of HS chains occurs with the assistance of GlcNAc transferase I (Fritz *et al.*, 1994) in the *cis-*, *medial-* and *trans-*Golgi (Prydz & Dalen, 2000) (Figure 1-12). Epimerization of GlcA to IdoA occurs with C5 GlcA-epimerase (Li *et al.*, 1997). Heparin/heparan sulfate GAGs are *N*-sulfated by the action of *N*-deacetylase/*N*-sulfotransferase (NDST) of which three have been cloned (Aikawa & Esko, 1999; Eriksson *et al.*, 1994; Hashimoto *et al.*, 1992; Humphries *et al.*, 1997; Orellana *et al.*, 1994). Heparin/heparan sulfate GAGs are *O*-sulfated on C3 and C6 on the GlcNAc by 3-*O*-sulfotransferase of which three multiple isoforms have been cloned (Shworak *et al.*, 1999) 6-*O*-sulfotransferase (Habuchi *et al.*, 1998), respectively. The C2 on the GlcA is sulfated by 2-*O*-sulfotransferase (Figure 1-14B) (Bai & Esko, 1996). Compared to heparin which has a high degree of sulfation (>1.5 sulfate groups per disaccharide), HSPGs have a low sulfation with 0.5-0.8 sulfate groups per disaccharide (Figure 1-14A) (Yanagishita, 1993).

Although heparin/HS GAGs share a similar backbone structure, heparin has a higher proportion of iduronic acid compared to HS which is comprised of GAGs containing more than 50% of unmodified *N*-acetyl glucosamine (Yanagishita, 1993) (Figure 1-14B). Connective tissue mast cells are the major cell type known to synthesize heparin (Prydz & Dalen, 2000; Yanagishita, 1993), whereas heparan sulfate GAGs are synthesized by different cells including vascular cells. Additionally, heparin is a potent anti-coagulant whereas HS GAGs do not show this activity (Yanagishita, 1993).

1.7.2.3 Keratan sulfate GAGs

Keratan sulfate (KS) GAGs such as those on osteoglycin/mimecan, differ from all other GAGs because the disaccharide repeats contain a galactose rather than the uronic acid (Schwartz, 2000) (Figure 1-14B). The other component of KS GAGs is GlcNAc. Either or both the galactose and the GlcNAc are sulfated at the C6 position, however in non-ocular tissues such as vascular smooth muscle, osteoglycin/mimecan may be found as a non-sulfated proteoglycan (Funderburgh *et al.*, 1997). The KS GAGs form two branches on a GlcA *O*-linked to a serine or alternatively the KS chain branches from mannose, GlcNAc, GlcNAc, *N*-linked to asparagine (Wight *et al.*, 1991). Another difference from other GAGs is that the final moiety of a KS GAG is sialic acid. Limiting the availability of UDP-galactose inhibits the synthesis of keratan sulfate GAGs and changes the sulfation pattern of chondroitin sulfate (Toma *et al.*, 1996).

1.7.2.4 Hyaluronan

Hyaluronan is a large molecular weight GAG (up to 1×10^4 kDa) containing repeating units of GlcNAc and GlcA (Figure 1-14B). Unlike other GAGs which are synthesized in the Golgi, hyaluronan is released from the cell surface as it is being synthesized by the hyaluronan synthase (HAS) enzyme. There are three known isoforms of HAS, of which HAS2 is known to be expressed and regulated in vascular SMCs (Meyer-Kirchrath *et al.*, 2004), however there is no known disease in which HA metabolism is the primary defect (Hascall *et al.*, 1991).

1.7.2.5 Regulation of glycosaminoglycan synthesis in vascular SMCs

Glycosaminoglycan (GAG) synthesis is a multi-factorial process involving the sequential addition of monosaccharides, isomerization of monosaccharides, and sulfation of different

positions on the monosaccharides. While the major enzymes involved in heparin/heparan sulfate and CS/DS synthesis have been established, their activity and regulation in vascular SMCs has not been described. Despite this, there have been several reports of various factors which alter the GAG synthetic process in vascular cells. Stimulation of vascular SMCs with PDGF increases the length of the GAGs and increases the 6:4-sulfation ratio of disaccharides however, it does not alter the composition of the GAGs (Schonherr et al., 1993). PDGF has also been shown to stimulate the synthesis of hyaluronan in proliferating human vascular SMCs (Papakonstantinou et al., 1995), however it remains to be determined whether or not this effect is due to regulation of HAS. In separate studies hyaluronan stimulation by PDGF and TGF-β1 in human fibroblasts was shown to involve the activation of protein kinase C (Suzuki et al., 1995).

Primate vascular SMCs treated with TGF-β1 synthesize proteoglycans with increased GAG length but no alteration in the sulfation pattern or composition of the GAGs (Schonherr et al., 1993) and these proteoglycans have shown increased binding to LDL (Little et al., 2002). Angiotensin II treatment of human aortic SMCs increases GAG length and charge and these changes increase the propensity to bind LDL (Figueroa & Vijayagopal, 2002). Vascular SMCs treated with oxidized LDL for 72 h synthesize proteoglycans with increased GAG length and these proteoglycans show an increased affinity for LDL (Chang et al., 2000). Human SMCs that have been stimulated to proliferate, increase proteoglycan synthesis. Two different studies have shown that proteoglycans from growth stimulated vascular SMCs show an increase in GAG length (Camejo et al., 1993), a decrease in heparan sulfate GAGs and an increase in the dermatan sulfate GAGs and no change in the sulfation pattern (Tao et al., 1997) however, these proteoglycans show increased binding to LDL compared to proteoglycans from quiescent SMCs (Camejo et al., 1993; Tao et al., 1997).

Under experimental conditions, a low level of sulfation is still sufficient to promote chain elongation, however GAG composition is altered. For example, incubation of fibroblasts with sodium chlorate, which inhibits the formation of PAPS (Rossi *et al.*, 1996), results in the production of undersulfated GAGs of similar if not greater GAG length than untreated cells (Greve *et al.*, 1988; Humphries & Silbert, 1988). Chlorate specifically inhibits 4-sulfation and consequently decreases the epimerization of CS to DS GAGs (Greve *et al.*, 1988). Similar effects of chlorate in human vascular SMCs has recently been described (Ballinger *et al.*, 2004). β-D-xyloside acts as a false initiator of chondroitin sulfate GAGs and its addition to cell cultures results in the secretion of core protein-free GAGs (Potter-Perigo *et al.*, 1992; Schwartz, 1977).

The core protein may also determine or influence the length of the GAGs. For example, *N*-terminal deletions in the decorin propeptide results in synthesis of shorter GAG chains in Chinese hamster ovary (CHO) and COS cells transfected with decorin (Oldberg *et al.*, 1996). Other factors which reduce vascular GAG length include glucosamine (Tannock *et al.*, 2002a) and calcium channel antagonists (Vijayagopal & Subramaniam, 2001), which induce changes that result in reduced binding to LDL. The mechanism of action of these agents to reduce GAG length is not known.

Studies in human arterial smooth muscle cells have shown that hyaluronan synthesis is inhibited by 8 mmol/L and 16.5 mmol/L glucose, 50-1000 μU insulin and stimulated by 1.25 ng/mL human growth hormone in the culture medium (Erikstrup *et al.*, 2001). In cultured human coronary SMCs, HAS2 expression is increased following treatment with prostacyclin mediated stimulation of cyclic adenosine monophosphate (cAMP) (Meyer-Kirchrath *et al.*, 2004).

Chondroitin sulfate
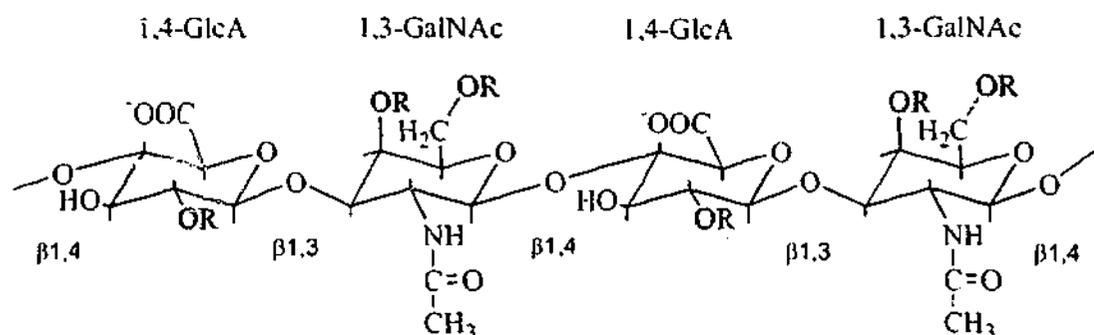
Dermatan sulfate
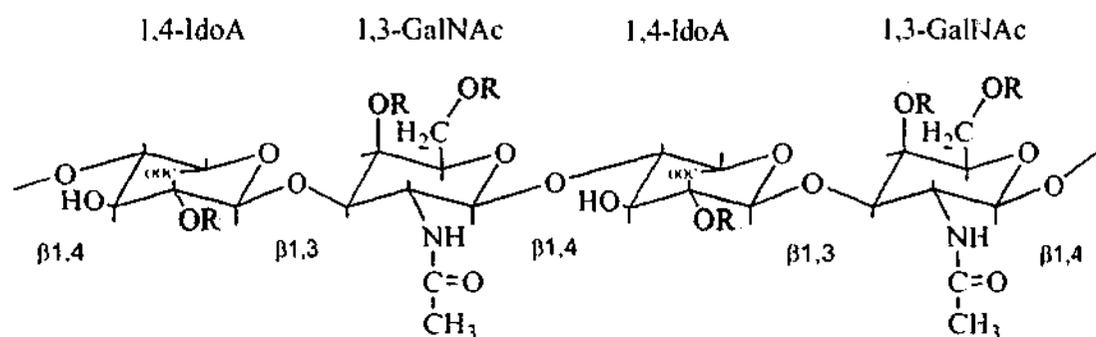
Heparan sulfate – low sulfation (0.5 SO$_4$/disaccharide)
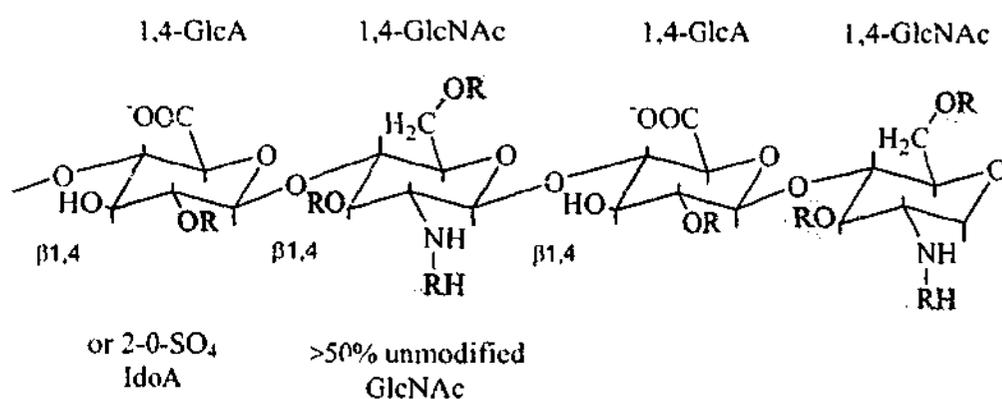
Figure 1-14A

Chemical structures of GAGs. Potential sites of sulfation (SO$_3^-$) are highlighted and represented by R. Adapted from (Hascall *et al.*, 1991; Jackson *et al.*, 1991; Prydz & Dalen, 2000).

Heparin - high sulfation (1.5 SO$_4$/disaccharide)

1,4-IdoA   1,4-GlcNAc   1,4-IdoA   1,4-GlcNAc

or 2-0-SO$_4$
GlcA

>80% N-SO$_4$
GlcNAc

Keratan sulfate

1,3-Gal   1,4-GlcNAc   1,3-Gal   1,4-GlcNAc

Hyaluronan

1,4-GlcA   1,3-GlcNAc   1,4-GlcA   1,3-GlcNAc
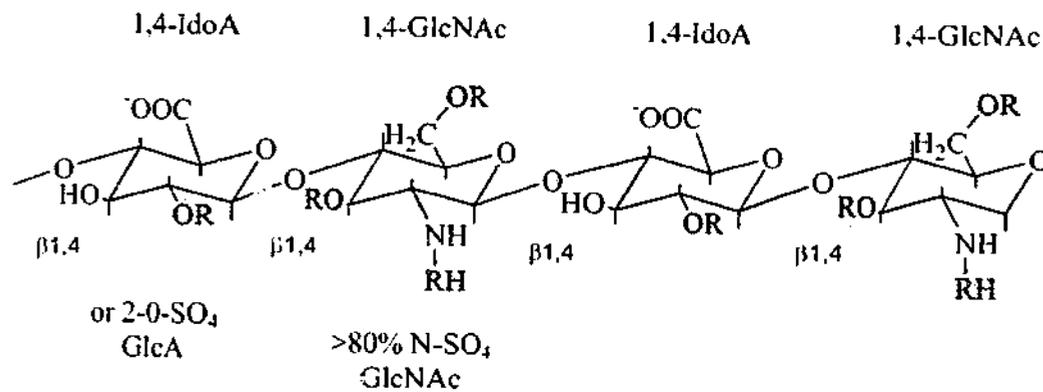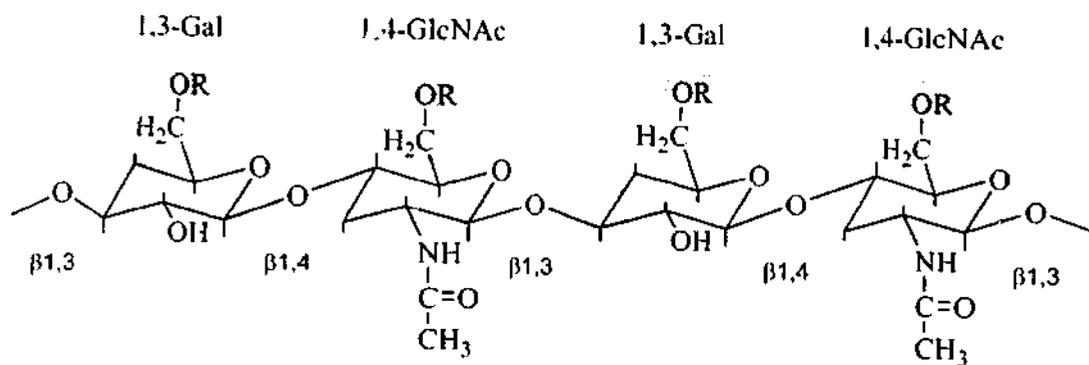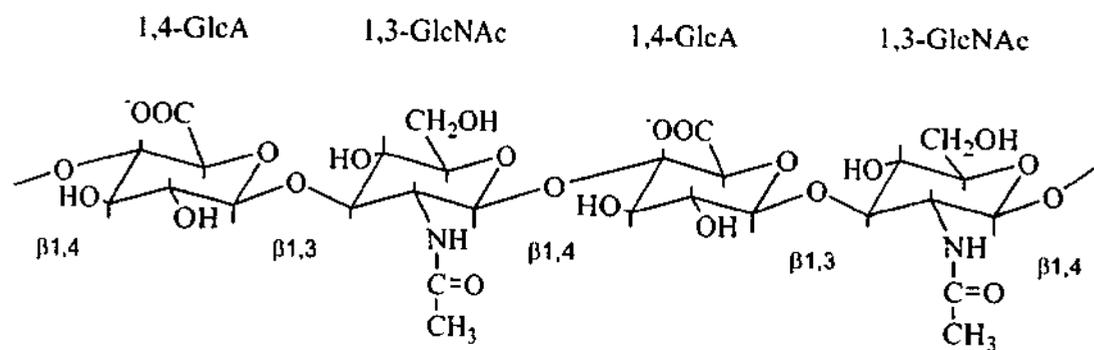
Figure 1-14B

Chemical structures of GAGs. Potential sites of sulfation (SO$_3^-$) are circled and represented

by R. Adapted from (Hascall *et al.*, 1991; Prydz & Dalen, 2000).

1.8 Rationale for the study of PPAR-α ligands in atherogenic processes

1.8.1 Known vascular targets of PPAR-α ligands

There has been early evidence that fibrates, in particular fenofibrate, has effects not directly related to its lipid-lowering action. In 1983, Pascal *et al* (Pascal *et al.*, 1983) showed that fenofibrate treatment of rat smooth muscle cells inhibited the re-initiation of DNA synthesis stimulated by the atherogenic growth factor, PDGF. In animal models and humans with hyperlipidemia, platelet aggregation is a well known phenomenon. In 1979, it was reported that fenofibrate decreased platelet aggregation in patients with hyperlipidemia (Renaud *et al.*, 1979). Recently, these observations have been extended by studies showing that the PPAR-α ligands, fenofibrate and WY-14,643 inhibit interleukin-6 (IL-6) and cyclo-oxygensase-2 (COX-2) inflammatory processes involved in the recruitment of macrophages to the atherosclerotic plaque (Staels *et al.*, 1998b). Fibrates improve hypertension and insulin sensitivity in humans (Kim *et al.*, 2003) and gemfibrozil has an additive effect with atorvastatin to improve lipid oxidation *in vitro* (Aviram *et al.*, 1998).

Staels and colleagues (Staels *et al.*, 1998b) have shown that the PPAR-α transcript is present, in three lines of human vascular smooth muscle, at levels comparable to the liver. Later, these receptors were shown to be expressed in other vascular cells such as endothelial cells (Inoue *et al.*, 1998) and macrophages (Ricote *et al.*, 1998). Additionally, PPAR-γ is also expressed in human vascular smooth muscle cells (Law *et al.*, 2000). It has been recognised by these two research groups that apart from the primary action to control blood lipids, the ligands for the PPARs have "pleiotropic" actions on processes involved in atherogenesis.

Various studies have indicated that PPAR-α activation is involved in the recruitment of inflammatory cells to the arterial lesion. PPAR-α decreases the expression and activity of human macrophage metalloproteinase-9, which degrades interstitial extracellular matrix,

(Chinetti *et al.*, 2003; Marx *et al.*, 1998; Shu *et al.*, 2000). PPAR-α activation reduces platelet activating factor expression in human monocytes and macrophages which could contribute to a reduction in leukocyte recruitment (Chinetti *et al.*, 2003; Hourton *et al.*, 2001). PPAR-α activation decreases the cellular levels of cholesteryl esters which accumulate in foam cells.

Animal studies have suggested that PPAR-α is involved in the atherogenic process (Tordjman *et al.*, 2001). When the apoE null mouse, a model of atherosclerosis, was crossed with the PPAR-α-null mouse, the lesion areas of three different vessels were significantly reduced compared to the same vessels from the PPAR-α$^{+/-}$apoE$^{-/-}$ mice (Tordjman *et al.*, 2001).

1.8.2 Unknown vascular targets of PPAR-α ligands - Do PPAR-α ligands affect vascular proteoglycan synthesis and binding to LDL?

Although proteoglycans are involved in multiple atherogenic processes, recent evidence has strongly implicated vascular proteoglycans for their role in trapping LDL in the vessel wall as a critical initiating event in atherogenesis (Skalen *et al.*, 2002; Williams & Tabas, 1995, 1998). Glycosaminoglycan elongation is associated with increased retention of LDL (Little *et al.*, 2002) and is stimulated by cell proliferation (Camejo *et al.*, 1993), PDGF (Schonherr *et al.*, 1993), TGF-β1 (Little *et al.*, 2002; Schonherr *et al.*, 1993), oxidized-LDL (Chang *et al.*, 2000) and free fatty acids (Olsson *et al.*, 1999). There are fewer examples of inhibition of GAG elongation including calcium channel antagonists (Vijayagopal & Subramaniam, 2001) and glucosamine (Tannock *et al.*, 2002a) which have been shown to reduce binding to LDL. We hypothesize that the modification of proteoglycans which reduces proteoglycan-LDL binding is a potential point of therapeutic intervention for the prevention of atherosclerosis. Our laboratory is investigating the pharmacological modulation of vascular proteoglycan

biosynthesis and structure as a pathway to new therapeutic agents for the treatment of atherosclerosis and for the delineation of beneficial vascular actions of existing cardiovascular and anti-diabetes drugs.

The use of gemfibrozil and fenofibrate in clinical trials such as VA-HIT (Rubins *et al.*, 1999) and DAIS (Diabetes Atherosclerosis Intervention Study Investigators, 2001) respectively, has suggested that these agents reduce the incidence of cardiovascular disease, especially in patients with diabetes. Furthermore, fenofibrate (Munro *et al.*, 1994) and gemfibrozil (Nigro *et al.*, 2002) inhibit human vascular SMC proliferation *in vitro*, independent of their triglyceride lowering effects. Whilst it has been shown that fibrate therapy favorably modifies the proteoglycan binding properties of human LDL (Wiklund *et al.*, 1996) by reducing small dense LDL (Anber *et al.*, 1997), it remains to be investigated whether or not these agents modify vascular smooth muscle cell proteoglycan biosynthesis and structure in a manner that would affect binding to atherogenic LDL.

The studies presented in this thesis are centered on the investigation of the PPAR-α ligand, fenofibrate on vascular proteoglycans and aim to:

1. Expand on the current understanding of the anti-atherogenic actions of PPAR-α ligands (e.g. fenofibrate) in vascular smooth muscle cells;

2. Determine whether or not the fibrates, via their action on PPAR-α or through a PPAR-α independent pathway, affect proteoglycan synthesis, structure and binding to LDL;

3. Investigate whether or not PPAR-α and -γ ligands alter glucose and glucosamine metabolism in vascular SMCs and how this affects metabolic labelling of proteoglycans;

4. Assess hyaluronan following the treatment of vascular SMCs with a PPAR-α and a PPAR-γ ligand;

5. Characterize the effects of growth factors, hormones and pharmacological agents (other than PPAR ligands) on proteoglycan synthesis, GAG elongation and sulfation;

6 Search for kinase sensitive GAG elongation enzymes and monosaccharide transporters in human vascular SMCs using proteomics.

Chapter 2: Methods

Cultured vascular smooth muscle cell as a model of the synthetic smooth muscle cell found in the intima of an atherosclerotic blood vessel

There are three main levels of research to study the vascular actions of therapeutic agents. Firstly, the *in vitro* analysis of the agents in cell cultures, secondly the effect of the agent in animal models of the disease and thirdly the effect of the drug in large, controlled clinical trials in the diseased human. To understand the full therapeutic potential of agents in humans it is important to understand the mechanisms of action *in vitro* and *in vivo*.

To study the vascular effects of PPAR ligands, we have used the smooth muscle cells (SMCs) derived from explants of the human internal mammary artery (IMA). It has been demonstrated that the smooth muscle cells, explanted from animal and human arteries retain their differentiated state at all times during the culture period (Campbell *et al.*, 1987; Ross, 1971), however multiply passaged cells take on the secretory characteristics of the vascular SMCs found in the intima *in vivo* (Schwartz *et al.*, 1986) and do not contract (Chamley-Campbell *et al.*, 1979).

Since there are many mechanisms contributing to the atherogenic cascade, we have chosen to use SMCs from the IMA for the reasons outlined below. Firstly there is a misconception that the vessel of origin determines the propensity for atherogenesis and will impact on behaviour *in vitro*. Prior to coronary artery bypass surgery, the internal mammary artery, radial artery and saphenous veins are relatively resistant to the development of atherosclerosis making them good choices for surgery. However, when these vessels are grafted they eventually develop atherosclerosis (Mautner *et al.*, 1992). A study has shown that the cellular and extracellular matrix composition of atherosclerotic plaques in saphenous vein used for CABG were similar to that of native coronary arteries after 7 years (Mautner *et al.*, 1992). Atherosclerosis occurs more frequently in specific sites of the vasculature, predominantly

points of curvature and flow dividers however, it is thought that this relationship depends on hemodynamic influences rather than some innate properties of the cells in those regions (Glagov *et al.*, 1988). Secondly, the *in vitro* environment of SMCs is different from their natural environment of the blood vessel where they are continuously contracting/relaxing to cope with blood flow and exhibit a contractile phenotype. Cells that have artificially undergone proliferation *in vitro* independent of blood vessel source may be equivalent to intimal cells *in vivo* because they have a synthetic phenotype and demonstrate pseudointimal characteristics (Chamley-Campbell *et al.*, 1979), often apparent in atherosclerotic vessels (Song *et al.*, 1998). Thirdly, smooth muscle cells reflect a reasonably consistent phenotype between blood vessels (Campbell *et al.*, 1987; Ross, 1971). SMCs from any blood vessel make proteoglycans, having minor differences in the quantity or the qualitative characteristics. Furthermore, our laboratory has shown that there is no difference between the growth inhibitory response to the PPAR-γ ligands, troglitazone, rosiglitazone and pioglitazone of SMCs from the IMA, saphenous vein and radial artery (de Dios *et al.*, 2003).

We have taken the approach of identifying factors which promote atherogenic behaviours in vascular smooth muscle cells (matrix and lipid accumulation) which will allow us to develop strategies for intervention in this process and lead to the development and improvement of new and current therapies for the specific action of reducing atherogenesis.

2.1 Materials

Mouse anti-human smooth muscle α-actin was from DAKO (Glostrup, Denmark), proliferating cell nuclear antigen antibody (PCNA) and human PPAR-α antibody (H-98) were from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). Mouse anti-human endothelial-nitric oxide synthase (e-NOS) was from Transduction Laboratories (Lexington, KY, USA). Horseradish peroxidase-conjugated to anti-mouse and anti-rabbit immunoglobulins was from DAKO (Carpinteria, CA, USA). Mouse monoclonal anti-vimentin, fenofibrate, dimethylsulfoxide (DMSO), human recombinant transforming growth factor (TGF)-β1, β-D-xylopyranoside (xyloside), platelet derived growth factor-BB (PDGF), insulin, diethylaminoethyl (DEAE)-sephacel, Sepharose CL-2B, Sepharose CL-6B, protease (*Streptomyces griseus*), chondroitinase ABC (*Proteus vulgaris*), chondroitinase AC (*Flavobacterium heparinum*) and chondroitinase B (*Flavobacterium heparinum*), heparitinase I (*Flavobacterium heparinum*), keratanase (*Pseudomonas sp.*), chondroitin sulfate, heparin, sodium chlorate and CHAPS were obtained from Sigma Chem. Co. (St Louis, MO, USA). GibcoBRL brand Dulbecco's modified Eagle Medium (DMEM) was from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from CSL (Parkville, VIC, Australia). Carrier free sodium [^{35}S]-sulfate (Product no. 64041, specific activity 1.38 Ci/μmol), [^{35}S]-methionine/cysteine (Tran^{35}S-label), [6-^{3}H]-D-glucosamine.HCl (Product no. 27021S, specific activity 25 mCi/μmol), [1-^{14}C]-glucosamine.HCl (Product no. 1005883, specific activity 53 mCi/mol) and cetylpyridinium chloride (CPC) were from ICN Biomedicals Inc. (Irvine, CA, USA). Whatman® chromatography paper, 3MM grade was from Whatman International Ltd (England, United Kingdom). Insta-gel Plus scintillation cocktail was purchased from Packard Bioscience B.V. (Groningen, The Netherlands). Poly-Prep® columns were from BIO-RAD (Hercules, CA). YM-3 microcon filters with

molecular weight cut-off 3000 Da were from Millipore Corp. (Bedford, MA, USA). 2-aminoacridone (AMAC) was purchased from Molecular Probes (Eugene, OR, USA). Unsaturated dermato/hyaluro-disaccharide standards were purchased from Seikagaku Corp. (Tokyo, Japan). Low molecular weight heparins, enoxaparin, octasaccharide and tetrasaccharide were a kind gift from Dr. André Uzan, Aventis-Pharma Laboratory, Centre of Research in Paris. Troglitazone was from Parke-Davis (Ann Arbor, MI). Glucose and lactate kits were from Roche Diagnostics (Mannheim, Germany). All other chemicals were of the highest grade commercially available and were purchased from Sigma Chem. Co. unless otherwise stated.

2.2   Explant of vascular smooth muscle cells

Smooth muscle cells were obtained from human blood vessels by the explant technique (Chamley-Campbell *et al.*, 1979; Sudhir *et al.*, 2001). Segments (1-2 cm) of the IMA, that would have otherwise been discarded during cardiac surgery at the Alfred Hospital, Melbourne, were cut longitudinally. The Human Ethics Committee of the Alfred Hospital approved the acquisition of tissue. The vessels were cleaned of fat and connective tissue, and the endothelium was removed by gently rubbing the inner layer of the vessel on a frosted glass plate. The media layer (containing only smooth muscle cells) was peeled away under a dissecting microscope. A segment of medial tissue was held under a sterile glass coverslip (9×22 mm) in a 90 mm petri dish containing 25 mmol/L glucose DMEM, supplemented with 10% FBS. The tissue was incubated at 37°C, 5% CO_2 in a water-jacketed incubator (Forma Scientific, Marietta, U.S.A) and was not disturbed for 1 week. The culture medium was changed 2-3 times per week. Cells that had migrated and proliferated from the tissue were passaged (1:3 split) at weeks 3-4. Stock cells from passage 5-7 were stored in liquid nitrogen

until required.

2.3 Immuno-cytochemical characterization of human vascular smooth muscle cells

Human vascular SMCs of the IMA from the 11th passage were grown to near confluence on a glass coverslip (22×22 mm) in 30 mm petri dishes in DMEM containing glucose (25 mmol/L) and FBS (10%). The cells were serum deprived (10 h) in low (5 mmol/L) glucose DMEM, supplemented with 0.1% serum, ensuring that cells were in the G_1 phase, i.e. quiescent. The glass slides were removed from the dishes and remaining media washed for 5-10 min with Dulbecco's phosphate buffered saline (PBS) containing 1.03 mmol/L $CaCl_2.2H_2O$, 2.68 mmol/L KCl 2.68, 1.47 mmol/L KH_2PO_4, 1.05 mmol/L $MgCl_2.6H_2O$, 137.9 mmol/L NaCl, 8.1 mmol/L Na_2HPO_4. The cells were fixed in acetone (10 min) and treated with Dulbecco's PBS containing 2% normal horse serum, to reduce non-specific binding of the primary antibody.

Immunoreactivity was detected by using the primary antibodies, mouse anti-human smooth muscle α-actin (1:500 in Dulbecco's PBS), vimentin (1:100 in Dulbecco's PBS), PCNA (1:100 in Dulbecco's PBS) and e-NOS (1:200 in Dulbecco's PBS). Horse radish peroxidase-conjugated to anti-mouse and anti-rabbit immunoglobulins (2 drops) was used as a secondary antibody, followed by the peroxidase substrate, diaminobenzidine tetrahydrochloride (DAB), to produce a brown end product at the site of the target antigen. Cell nuclei were counter stained with haematoxylin.

2.4 Western blot for PPAR-α

PPAR-α protein was detected in human vascular SMCs as pr ously described by others (Staels et al., 1998b) with some minor variations to the protocol. Human vascular SMCs were

grown to confluence on 90 mm plates and serum deprived for 48 h prior to treatment with control medium containing 0.1% DMSO or medium containing fenofibrate (30 μmol/L) or troglitazone (10 μmol/L). As a positive control, the protein from cultured hepatocytes (HepG2 cell line) was also tested for PPAR-α, alongside the protein from human vascular SMCs. After a 24 h treatment, the cells were washed with Dulbecco's PBS. Cells were lysed in 100 μL of a buffer containing 1% Triton X-100, 0.15 mol/L NaCl, 0.01 mol/L Tris pH 7.5, 1 mmol/L EDTA, 1 μmol/L EGTA pH 9.0, 0.5% NP-40, 80 μmol/L PMSF, 2 mmol/L ω-amino caproic acid and 0.1 mmol/L benzamidine. The cells were scraped from the dish and collected into a 1.5 mL centrifuge tube. Samples were passed through a fine needle followed by centrifugation at 1000 rpm for 10 min. The supernatant was collected and assayed for protein content using the bicinchoninic acid (BCA) Colorimetric Assay (Pierce, Rockford, IL, USA). Protein (50 μg) samples were separated by electrophoresis on a 10% separating gel with a 4% stacking gel (Laemmli, 1970). Benchmark™ Prestained protein ladder (Invitrogen, CA) was used as a reference for protein molecular weight. Following electrophoresis, proteins were transferred to nitrocellulose filters (Immobilon™, Millipore, Bedford, MA, USA) at 4°C, overnight. The blots were washed twice with Tris buffered saline (TBS)-Tween, containing 10 mmol/L Tris, 0.15 mol/L NaCl and 0.5% Tween-20. Non-specific binding was blocked by immersing the membrane in TBS-Tween containing 5% non-fat dry milk (NFDM) for 1 h at 25°C. Four 5 min washes in TBS-Tween were performed at 25°C prior to overnight incubation of the blot at 4°C with the rabbit polyclonal anti-human PPAR-α primary antibody at a dilution of 1:200 in 5% NFDM/TBS-Tween. The blot was washed four times for 5 min with TBS-Tween and then incubated for 1 h at 25°C with the secondary antibody, horse radish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences, England, UK) diluted 1:1000 in 5% NFDM/TBS-Tween. The blot was washed four times in TBS-Tween, and the reaction

was detected using enhanced chemiluminescence (ECL, Amersham Biosciences, England, UK) according to the manufacturer's instructions and 30 sec to 2 min exposures to x-ray films.

2.5 Analysis of proteoglycan biosynthesis

Generally, the analysis of proteoglycan synthesis was performed with human vascular SMCs seeded at 5×10^4 cells per well in a 24-well plate (Becton Dickinson) in DMEM containing 5 mmol/L glucose and 10% FBS which was replenished the following day. The cells were grown to confluence, and then serum deprived in DMEM containing 0.1% FBS for 48 hours. Smooth muscle cells were treated with control medium containing 0.1% FBS/0.1% DMSO or treatment medium with fenofibrate (30-50 µmol/L), in the presence or absence of TGF-β1 (2 ng/mL). To assess the effect of fenofibrate treatment on the GAG synthesizing mechanisms, separate media solutions with and without fenofibrate were prepared with β-D-xyloside (0.5 mmol/L). For the dose response studies, SMCs were treated with fenofibrate (0.3-50 µmol/L) or fenofibric acid (1-300 µmol/L) in the presence and absence of TGF-β1 (1 ng/mL) or PDGF (50 ng/mL). Vascular SMCs were treated with GW7647 (0.01-3 µmol/L), in the presence and absence of TGF-β1 (2 ng/mL) or PDGF (50 ng/mL) with insulin (1 µmol/L). The growth factor and hormone dose response curves were obtained following the treatment of vascular SMCs with TGF-β1 (0.03-3 ng/mL), PDGF (1-300 ng/mL) and angiotensin II (1-300 nmol/L). To study the effects of calcium channel antagonists on proteoglycan synthesis, vascular SMCs were treated with felodipine, verapamil or Bay K 8644 each at 3 µmol/L, in the presence and absence of TGF-β1 (1 ng/mL) or PDGF (50 ng/mL).

For the various treatments, vascular SMCs were metabolically labelled with 50 µCi/mL carrier free sodium [^{35}S]-sulfate for 24 h. Parallel plates were established without [^{35}S]-sulfate

to count cells on the Z2 Coulter counter (Coulter Corp. Miami, FL, USA). Aliquots (50 µL) of the media and cell layer samples were spotted in duplicate on 30×15 mm rectangles of Whatman® 3MM filter paper. The paper was washed five times in CPC solution (1% CPC, 0.05 mol/L NaCl). Following drying, the paper from each sample was placed in scintillation fluid and counted on a liquid scintillation analyzer (Packard, Meriden, CT, USA). These counts represented total proteoglycans.

2.6 Isolation and concentration of proteoglycans by ion-exchange chromatography

The strong negative charge on proteoglycan GAG chains makes them easy to separate from other molecules by ion-exchange chromatography (Prydz & Dalen, 2000). Following similar protocols described previously (Little *et al.*, 2002), Poly-Prep® columns were filled with 500 µL of DEAE-sephacel (50%$^v/_v$) equilibrated in low salt buffer containing 8 mol/L urea, 0.25 mol/L NaCl, 20 mmol/L disodium EDTA and 0.5% Triton X-100. The columns were washed with low salt buffer and the media of 3 identical treatments was pooled and applied to the column. The columns were washed with low salt buffer (5×7 mL) to remove non-proteoglycan associated radioactivity. Proteoglycans were eluted (5×0.3 mL) with high salt buffer containing 8 mol/L urea, 3 mol/L NaCl, 20 mmol/L disodium EDTA and 0.5% Triton X-100. An aliquot from each fraction (10 µL) was counted. Fractions containing >15% of the total radioactivity, generally the first two fractions, were pooled. An aliquot (10 µL) of the pooled fractions was re-counted.

2.7 Assessment of glycosaminoglycan electrophoretic mobility by SDS-PAGE

Samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) by equal counts so that the intensity of the bands on the gel would be relatively the same between each treatment. Samples containing $20\text{-}30\times10^3$ cpm were mixed with water and chondroitin sulfate (250 µg/mL) was added as a "cold carrier". Ice cold ethanol (95%) containing potassium acetate (1.3%; 700 µl) was added to each sample, to precipitate the proteoglycans and remove the urea. Following brief vortexing, the samples were stored at -20°C for 1 h. The samples were microfuged (14×10^3 rpm) at 4°C for 10 min. The supernatant was discarded and the pellets were resuspended in dH$_2$O (200 µl) and reprecipitated with the ethanol-acetate solution (700 µl). Following brief vortexing, the samples were stored at -20°C for 1 h. The samples were microfuged (14×10^3 rpm) at 4°C (10 min). The supernatant was discarded and the pellets were left to air dry. The dry pellets were resuspended in 8 mol/L urea buffer (20 µl).

Proteoglycans were separated on 4-13% linear gradient separating gels and proteoglycans plus xyloside-initiated GAGs on 4-20% linear gradient separating gels, each with a 3% stacking gel. For the estimation of the apparent relative masses (M$_r$S) of proteoglycans, ^{14}C high molecular weight standards (Life Technologies, Rockville, MD, USA) or SeeBlue Pre-Stained Standard (Invitrogen Life Technologies, Carlsbad, CA, USA) molecular weight standards were used. The radiolabelled proteoglycans and standard proteins were exposed to an imaging plate (Fuji Photo Film Co., Tokyo, Japan) for 3 days and the result visualized by a Bio-imaging analyzer BAS-1000 and an Apple Macintosh computer with MacBas software (Fuji Photo Film Co., Tokyo, Japan). Gel images were analyzed using Image Quant 5.1

(Molecular Dynamics, Sunnyvale, CA, USA).

2.8 Size exclusion chromatography

2.8.1 Column calibration, calculation of K_{av} and conversion of K_{av} to molecular weight

The Sepharose CL-2B and CL-6B columns were calibrated by preparing a sample containing a large molecule ($[^3H]$-DNA) and a small molecule (free $[^{35}S]$-sulfate) and counting the fractions on the liquid scintillation counter. The chromatograms were plotted (Figure 2-1) and the column constant was calculated by dividing the fraction number with the highest ^{35}S count (V_t) by the fraction number with the highest ^3H count (V_o).

Following separation of a sample, fractions were assigned a distribution coefficient (K_{av}) to standardize the data. The K_{av} was calculated using the following equation (Fischer, 1980):

$$\left[\frac{\text{Fraction number} - V_o}{(V_t - V_o)} \right]$$

Each sample included free $[^{35}S]$-sulfate and following separation by size exclusion chromatography the V_t was assigned to the fraction with the highest count of free $[^{35}S]$-sulfate and the column constant was used to calculate the V_o.

To convert the K_{av} values from a CL-6B column to molecular weight, the data of Wasteson (Wasteson, 1971) was graphically represented (Figure 2-2) and an equation for the line was obtained:

$$\text{Log MW} = (-2.27 \times K_{av}) + 5.45$$

where MW is the molecular weight in Daltons.

Calibration of Sepharose CL-2B column
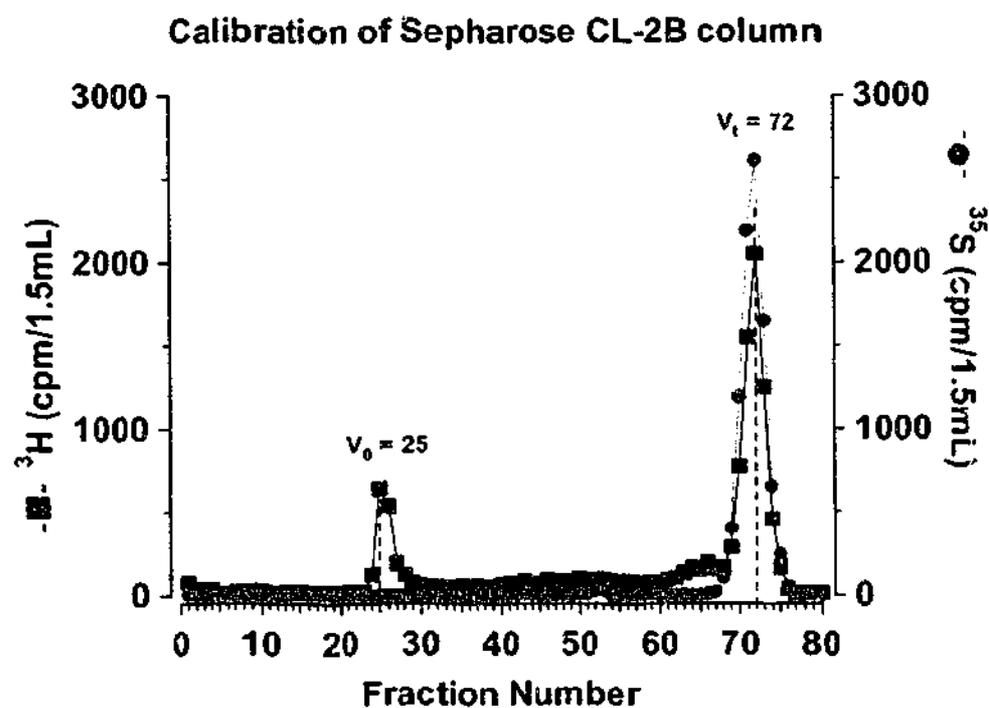
Figure 2-1

Calibration of a Sepharose CL-2B column. A sample containing [^{3}H]-DNA and free [^{35}S]-sulfate was applied to a 100 mm CL-2B column. Fractions ($\approx$1 mL) were collected every 4.4 min. Each fraction was combined with scintillation fluid and counted for ^{3}H (blue squares) and ^{35}S (red circles) on a liquid scintillation counter and the results plotted. The column constant was calculated by dividing the V_t by the V_0, and in this example is equal to 2.88.

A Wasteson - raw data

B Wasteson - transformed data

Log MW = (-2.27 x K_{av}) + 5.45
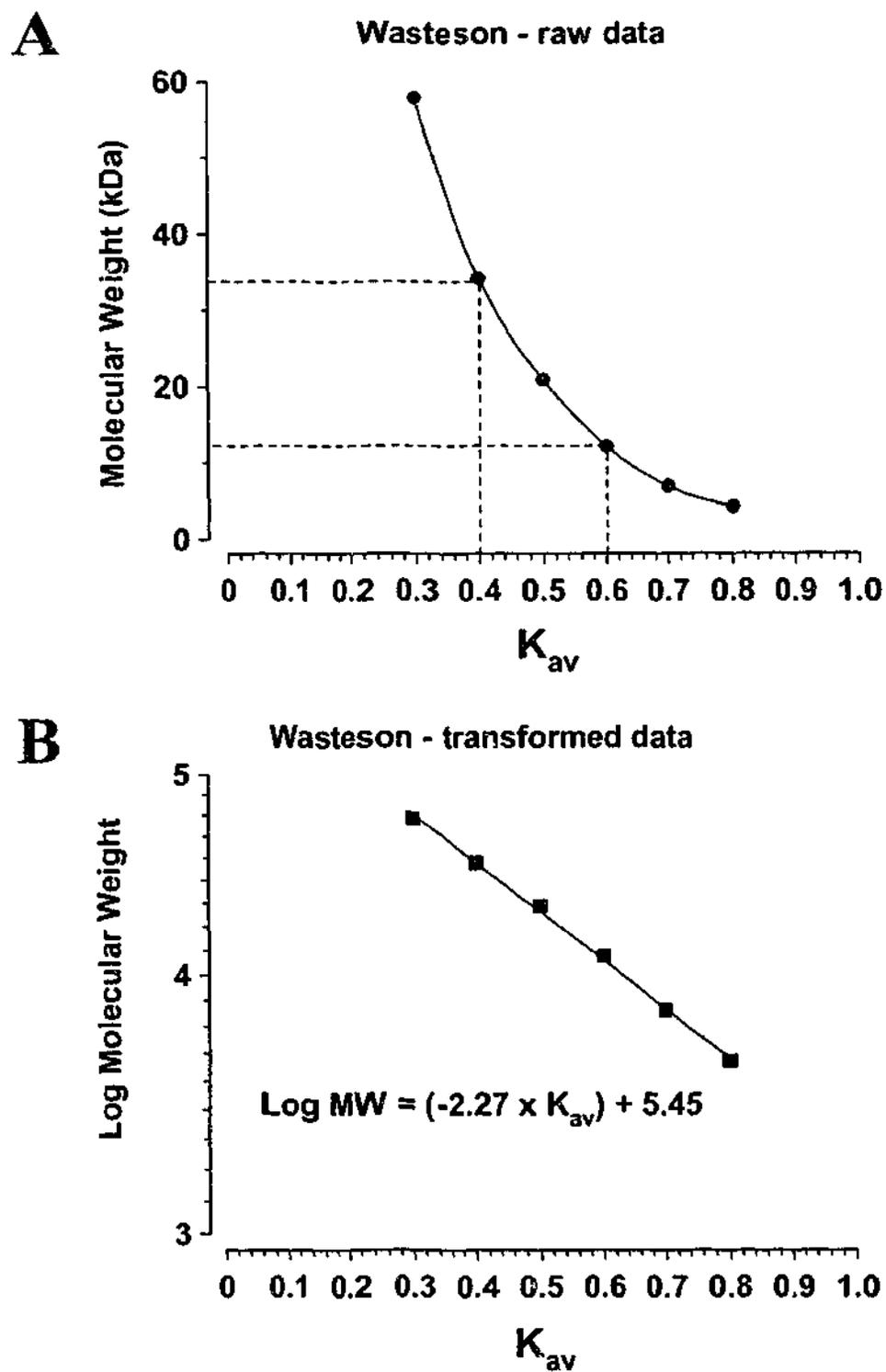
Figure 2-2

Conversion of K_{av} to molecular weight. A) The raw data from Wasteson (Wasteson, 1971) was used to create B) a linear transformation which converts the K_{av} to molecular weight. The conversion of K_{av} to molecular weight may only be accurate for K_{av} values between 0.3-0.8, which is a molecular weight range of ≈5-58 kDa. Data in B was fitted with a linear regression using "P Fit" in Fig.P Version 2.98.

2.8.2 Analysis of vascular proteoglycans by size exclusion chromatography

We used Sepharose CL-2B to size intact proteoglycans and Sepharose CL-6B to size the smaller cleaved chains and xyloside-initiated GAGs (Chang *et al.*, 2000; Little *et al.*, 2002). To obtain the required amount of sample, human vascular SMCs were cultured at 5×10^5 cells per 60 mm plate following a growth protocol described under Section 2.5. Human vascular SMCs were pre-treated (6 h) with control medium (0.1% DMSO) or fenofibrate (30 µmol/L), in the presence and absence of xyloside (0.5 mmol/L). Following the pre-incubation period, the treatments were removed and fresh drug was replaced followed by the addition of TGF-β1 (2 ng/mL) and 50 µCi/mL [^{35}S]-sulfate for 16 h. After the treatment and labelling period, the culture medium was purified by DEAE (refer to section 2.7). The intact proteoglycan samples containing $6-10 \times 10^4$ cpm and free [^{35}S]-sulfate label were separated on a 100 mm CL-2B column in the presence of 1% phenol red and a buffer containing 4 mol/L guanidine, 0.1 mol/L sodium sulfate, 0.1 mol/L Tris, 2.5 mmol/L disodium EDTA, 0.5% Triton X-100, pH 7.0 (guanidine buffer). To cleave off the GAG chains, intact proteoglycans were ethanol precipitated and reconstituted in a solution containing 1 mol/L sodium borohydride and 0.05 mol/L sodium hydroxide and incubated at 45°C for 24 h. The β-elimination reaction was terminated by neutralization with glacial acetic acid (Little *et al.*, 2002). Cleaved GAG chains and xyloside preparations containing $6-10 \times 10^4$ cpm and free [^{35}S]-sulfate label were applied to a 100 mm Sepharose CL-6B column in the presence of 1% phenol red and guanidine buffer. The samples from either the CL-2B or the CL-6B were eluted with guanidine buffer and the fractions collected with an automated fraction collector (Amersham Pharmacia Biotech). Each fraction was combined with Instagel Plus and then analyzed on a liquid scintillation analyzer (Packard, Meriden, CT, USA). The V_t was assigned to the fraction with the highest free [^{35}S]-sulfate count and fraction numbers were converted to K_{av}. For cleaved GAG chains and

xyloside-initiated GAGs, we used the data of Wasteson, as processed in Figure 2-2B, to convert K_{av} to molecular weight to give both more intuitive presentation of the data and allow for calculation of the changes in terms of number of monosaccharides on GAGs.

2.9 Fluorophore Assisted Carbohydrate Electrophoresis (FACE)

2.9.1 Theory of FACE

Fluorophore assisted carbohydrate electrophoresis is used to analyze the disaccharide composition of glycosaminoglycans. The proteoglycan sample is subjected to digestion with enzyme(s) specific to the GAGs in the preparation, to yield disaccharides in the unknown material (Figure 2-3). Released disaccharides are then quantitatively and stoichiometrically (1:1) labelled with a fluorophore and resolved by high percentage acrylamide (20%) gel electrophoresis. The GAG disaccharides are identified by comparison of their migration with fluorophore tagged GAG disaccharide standards. Gel images are captured by fluorescence.

2.9.2 Procedure of FACE

The procedure for FACE was performed as described by Calabro *et al* (Calabro *et al.*, 2000a; Calabro *et al.*, 2000b; Plaas *et al.*, 2001). Vascular SMCs were cultured on 90 mm plates at a density of $8-10 \times 10^5$ cells per plate, with a culture protocol identical to that described for the analysis of size. Cells were treated with fenofibrate (30 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) for 24 h. In separate experiments, SMCs were treated with sodium chlorate (0.5-5 mmol/L) in the presence of TGF-β1 (2 ng/mL) for 24 h for the FACE validation studies. To assess the specific activity of glucosamine incorporation into disaccharides, confluent cultures on 75 cm^2 flasks were treated with fenofibrate (30 μmol/L) or troglitazone (10 μmol/L) in DMEM containing either 5 mmol/L glucose or 25 mmol/L

glucose. Proteoglycans were isolated from the medium of SMCs using DEAE-Sephacel ion-exchange and fractions eluted in a buffer containing 8 mol/L urea, 3 mol/L NaCl and 0.5% CHAPS. The fractions with the highest absorbance at 280 nm (and therefore the highest protein content) were dialyzed into high quality water and subsequently dried in a vacuum concentrator. To degrade the core proteins, 3.3 U/mL protease (300 μL) reconstituted in 100 mmol/L ammonium acetate pH 7.0 was added per sample of proteoglycan for 18-24 h at 60°C. The protease was inactivated by heating at 100°C for 20 min. Protein digestion products were removed by centrifugation (13,200 rpm, 5 min). GAGs were precipitated with ethanol so that the final concentration was 75%. GAGs were reconstituted in water (100 μL) and assayed for GAG content using the DMMB assay (Templeton, 1988). Aliquots containing 1-5 μg of GAG were digested with chondroitinase ABC (50 mU/mL) for 16 h at 37°C in a buffer containing 50 mmol/L ammonium acetate, pH 7.3. Undigested material was removed by using a YM3 Microcon filter and the filtrate lyophilized. Lyophilized samples were labelled with 50 mmol/L 2-aminoacridone (5 μL) for 16 h, at 37°C in a buffer containing 0.5 mol/L cyanoborohydride. Samples were cooled, mixed with 37.5% glycerol (10 μL) and an aliquot (3-5 μL) separated on 20% acrylamide gels at a constant current of 30 mA/gel and 500 V. Images were captured using the Glyko Imager (ProZyme, San Leandro, CA, USA) and quantitation of bands was performed using the Glyko software (ProZyme, San Leandro, CA, USA).
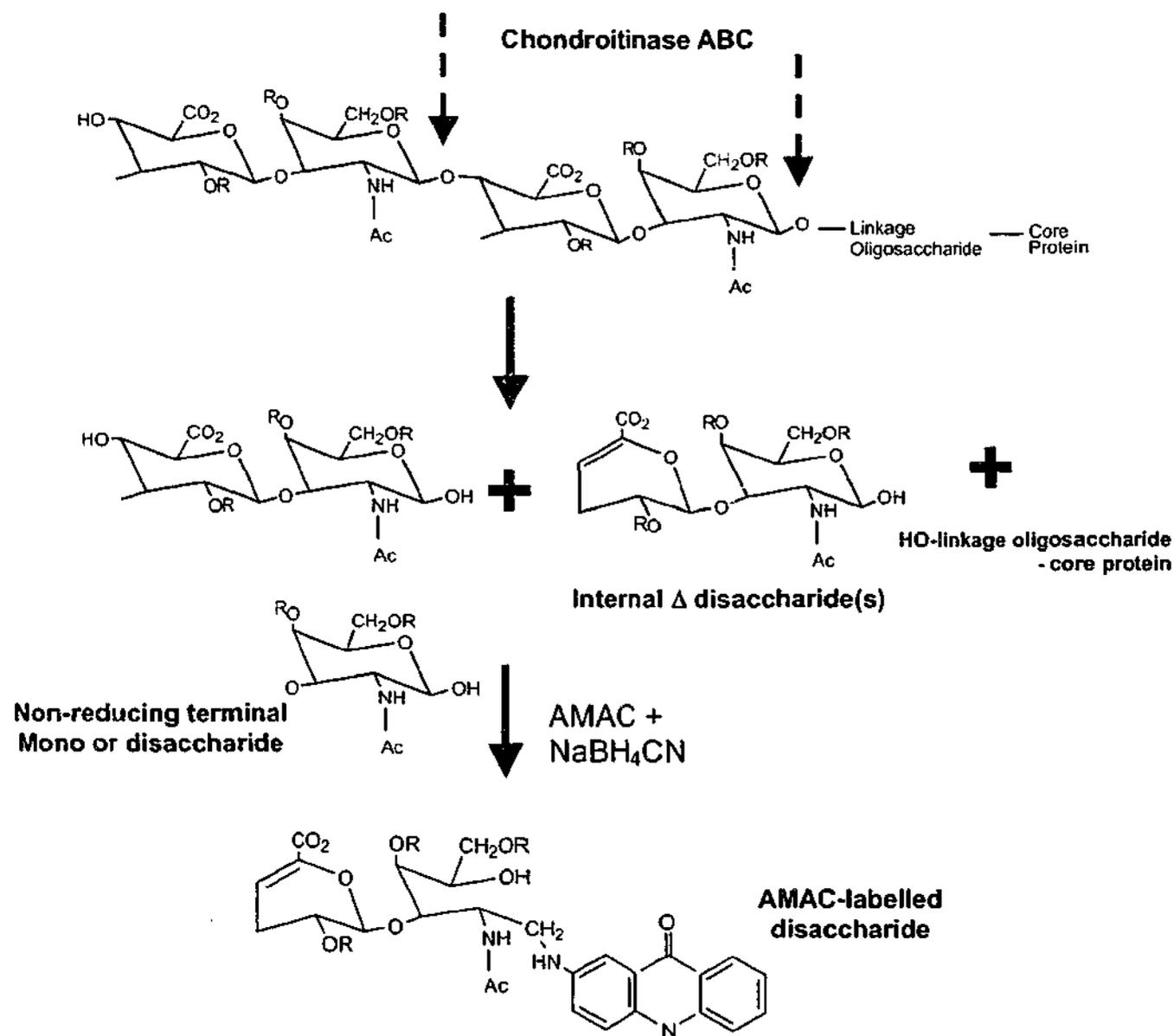
Figure 2·3

Chemical reactions involved in the preparation of samples for FACE analysis. Chondroitinase ABC digests chondroitin/dermatan sulfate GAG chains after the linkage region, producing saturated disaccharides and a non-reducing terminal moiety. Each lyase product contains a free reducing end that can be coupled to a fluorescent tag (*2-aminoacridone*) by reductive amination. The fluorotagged products can be resolved into discrete bands by electrophoresis on high percentage polyacrylamide gels. Gels are illuminated with UV light. $R=SO_3^-$. Adapted from Plaas *et al* (Plaas *et al.*, 2001).

2.10 Characterization of the predominant glycosaminoglycans in human vascular smooth muscle cells by enzymatic digestion and SDS-PAGE

Proteoglycans were metabolically labelled with $[^{35}S]$-sulfate, isolated and concentrated from the culture medium as described in Section 2.6. An aliquot of proteoglycans containing 20,000 cpm was ethanol precipitated at -20°C overnight and the proteoglycans were pelleted in a bench top centrifuge (13,200 rpm, 10 min, 4°C). Digestions were performed according to the manufacturer's instructions. To digest chondroitin sulfate/dermatan sulfate (CS/DS) chains, 2.5 U/mL Chondroitinase ABC was reconstituted in a buffer containing 20 mmol/L Tris-HCl pH 8.0 and 0.1% BSA, and was used at a final concentration of 42 mU/mL in a digestion buffer containing 40 mmol/L Tris-HCl pH 8.0, 40 mmol/L sodium acetate and 0.01% bovine serum albumin (BSA). Chondroitin AC, which digests CS chains, was reconstituted in a buffer containing 20 mmol/L Tris-HCl pH 7.3 and 0.1% BSA and was used for GAG digestion at a final concentration of 42 mU/mL in a buffer containing 40 mmol/L Tris-HCl pH 7.3, 40 mmol/L sodium acetate and 0.01% BSA at 37°C for 16 h. To digest DS chains, chondroitinase B was reconstituted in a buffer containing 50 mmol/L Tris-HCl pH 8.0 and 0.05% BSA and used at a final concentration of 25 mU/mL in the same buffer. Heparitinase was reconstituted in a buffer containing 100 mmol/L sodium acetate, 10 mmol/L calcium acetate, pH7.0 and was used at 25 mU/mL in the same buffer. Keratanase was reconstituted in 10 μmol/L Tris-HCl, pH 7.4 and used at a final concentration of 25 mU/mL in the same buffer. All enzymatic reactions occurred at 37°C for 16 h and included a proteoglycan sample with no enzyme. Following ethanol precipitation, remaining proteoglycans were separated on 4-13% linear gradient SDS-gels and visualized by a phosphorimager as described in Section 2.8. Computer analysis (MacBas Version 1.0) was used to quantify undigested material.

2.11 Gel Mobility Shift Assay to quantify LDL binding to proteoglycans

To analyze the binding interaction of proteoglycans with apolipoprotein on LDL, we used the gel mobility shift assay (Hurt-Camejo et al., 1998; Little et al., 2002). Cells were cultured at 5×10^5 cells per 60 mm plate following a growth protocol described under Section 2.5. Human vascular SMCs were metabolically labelled with 40 μCi/mL [^{35}S]-methionine/cysteine Tran^{35}S-label (ICN Biomedicals). Following isolation and concentration by DEAE-Sephacel, proteoglycans were dialyzed into a physiological buffer containing 20 mmol/L MOPS, 140 mmol/L NaCl, 5 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, pH 7.4. LDL cholesterol was isolated from human plasma using the sequential density gradient ultracentrifugation method as previously described (Heinecke et al., 1986). Various concentrations of human LDL (0-500 μg/mL) were each incubated with 1500 cpm of radiolabelled proteoglycans for 1 h at 37°C. Samples were run on a 0.8% agarose gel in a buffer containing 20 mmol/L MOPS, 2 mmol/L CaCl$_2$, 4 mmol/L MgCl$_2$, pH 7.0 at 4°C for 2-3 h at 60 V. The gels were fixed in 75% ethanol containing 1% CPC. Dry gels were exposed to a phosphorimaging plate for 3 days and scanned on a phosphorimager. Computer analysis (MacBas Version 1.0) was used to quantify the bound and free proteoglycans on the gels.

2.12 Analytical ion-change chromatography to assess the charge of hyaluronan and proteoglycans

To analyze the charge of proteoglycans and hyaluronan we used analytical ion-exchange chromatography as previously described with some modifications (Chang et al., 1983). Monkey vascular SMCs from the thoracic aorta were cultured at 5×10^4 cells per 60 mm plate in DMEM containing 5 mmol/L glucose and 5% FBS. Cultures were grown to confluence and

serum deprived for 48 h in DMEM containing 0.1% FBS. Cells were pre-treated for 6 h with medium containing 0.1% DMSO or fenofibrate (30 µmol/L). Treatment medium was removed and replaced with fresh treatments in the presence and absence of TGF-β1 (2 ng/mL). The cells were metabolically labelled with 100 µCi/mL [^{35}S]-sulfate and 10 µCi/mL [^{3}H]-glucosamine for 24 h. The unincorporated label in the culture medium was removed using a Sephadex-G50 column; a plastic serological pipette filled with a 4-mL bed of Sephadex-G50, equilibrated in a buffer containing 8 mol/L urea and 0.1 mol/L NaCl. Fractions (1.0 mL) were collected and the large metabolically labelled molecules eluted in the fourth fraction. An aliquot of the sample containing 30-40×10^3 cpm was loaded onto an equilibrated DEAE-Sephacel column, prepared with 3-5 mL of DEAE-Sephacel (50%$^v/_v$ in 8 mol/L urea/0.1 mol/L NaCl) in a Poly-Prep® column (Figure 2-4). The column was washed with 5 mL of 8 mol/L urea/0.1 mol/L NaCl, followed by a continuous 0.1 mol/L to 0.8 mol/L NaCl gradient (Figure 2-4). The total volume used was 20 mL and fractions (250-400 µL) were collected at a flow rate of 0.2-0.4 mL/min. An aliquot (200 µL) of each fraction was combined with scintillation fluid and analyzed on a liquid scintillation counter. The NaCl gradient was established by testing the conductivity of every 3rd fraction using a conductivity meter.
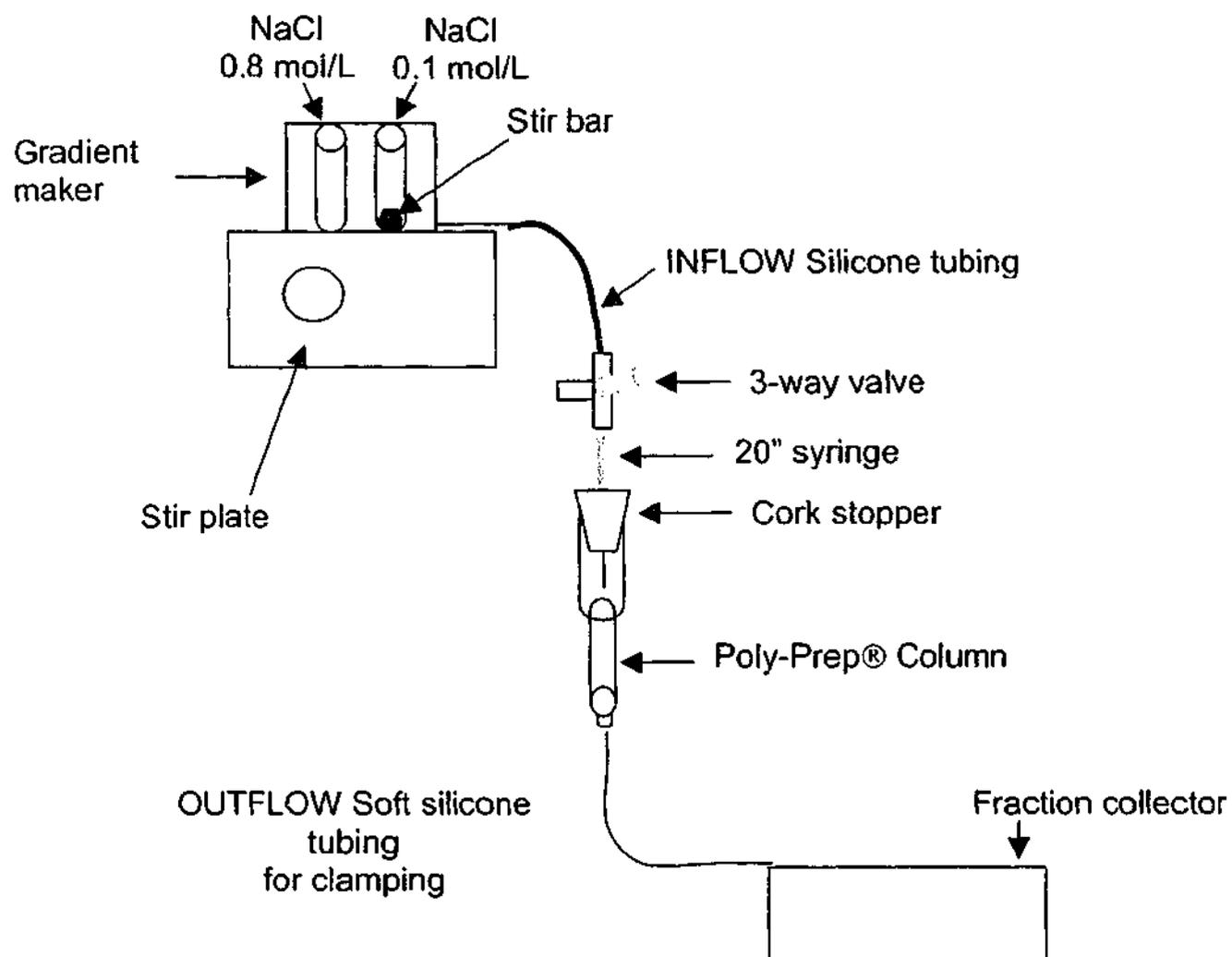
Figure 2-4

Apparatus and set-up of the analytical ion-exchange column. A gradient maker was used to elute the sample from the DEAE-sephacel column with 0.1-0.8 mol/L NaCl. Fractions (250-400 µL) were collected automatically.

2.13 Hyaluronan mass assay by hyaluronan binding region (HA-br) colorimetric assay

2.13.1 Principle

The core protein of the proteoglycan, versican, has a hyaluronan (HA) binding region (br) (refer to Chapter 1, Figure 1-10B). Soluble HA (variable) present in the tested samples and the standard binds to biotinylated proteoglycan (b-PG) through the HA-br of aggrecan core protein. The remaining b-PG that has not bound to soluble HA, binds to excess plastic/BSA-immobilized HA (constant). The amount of HA bound to the plastic is determined by a colorimetric reaction mediated by streptavidin-peroxidase. The amount of HA originally present in the samples and standards are indirectly determined by a negative competition with HA on the plastic.

2.13.2 Methodology

The HA-br method was initially described by Fosang *et al* (Fosang *et al.*, 1990) and has been used to measure HA from fibroblasts (Wilkinson *et al.*, 2004). This method was used with minor modifications. The culture medium from SMC cultures treated with PPAR ligands, fenofibrate (30 µmol/L) and troglitazone (10 µmol/L) was digested with 100 µg/mL protease at 60°C for 16 h. The protease was inactivated by heating at 100°C for 10 min. Hyaluronan standards (18.75-1200 ng/mL) were prepared in sterile PBS. Samples and standards were combined with 5 µg/mL biotinylated proteoglycan (b-PG) and incubated at room temperature for 1 h. A 96-well plastic plate (NUNC) was coated with 100 µL of 200 µg/mL hyaluronan conjugated to BSA (HA-BSA) for 1-2 h at room temperature. Each well was washed with PBS (200 µL), 3-4 times. A solution containing 10% FBS in PBS was used to block the non-specific binding for 1-2 h at room temperature. The calf serum was removed by shaking

out. The standards and the samples (60 μL each) were added and incubated overnight at 4°C. The wells were washed with PBS (200 μL) 3-4 times, followed by the addition of 2 μg/mL Streptavidin-labelled peroxidase (60 μL) for 30 min. Following washing the colour reagent, 0.03% H_2O_2/0.5 mg/mL 2,2'azinobis 3-ethyl-benzthiozoline sulfonic acid/0.1 mol/L Na Citrate pH 4.2 (60 μL), was added for up to 30 min at room temperature. The absorbance was read at 405 nm on an automated plate reader (BIO-RAD, Hercules, CA). The HA standards were plotted as a function of the log HA concentration to determine HA in the unknown samples. A scheme for the HA-br assay is shown in Figure 2-5.

2.14 Measurement of glucose utilization and lactate production

To measure glucose consumption and lactate production in the culture medium of human vascular SMCs, we used the hexokinase/NADPH and lactate oxidase and peroxidase reactions, respectively. Confluent, serum deprived human vascular SMCs were treated with fenofibrate (50 μmol/L) or troglitazone (10 μmol/L) for 24 hours. Media was removed and analyzed for glucose by the photometric Hexokinase/NADPH reaction and lactate was analyzed using a colorimetric reaction with lactate oxidase and peroxidase (Roche Diagnostics, Mannheim, Germany). Reaction endpoints were detected using a COBAS FARA Analytical System (Roche, Switzerland). Measurements were adjusted to cell number and time.
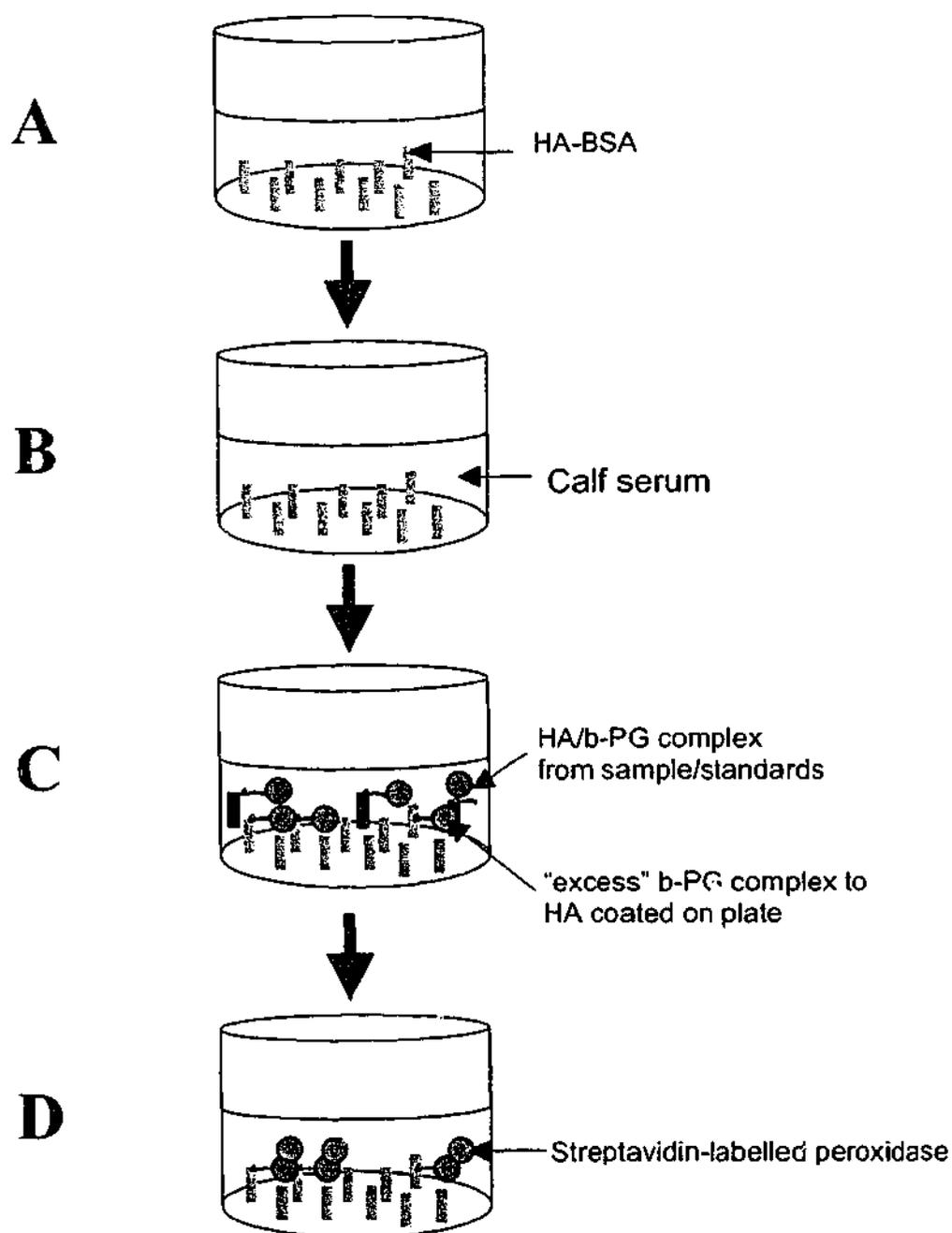
Figure 2-5

Schematic representation of the HA-br assay. A) A plastic well is coated with excess hyaluronan and B) non-specific sites are blocked with calf serum. C) A sample or standard with HA bound to biotinylated proteoglycan (b-PG) is added and any unbound b-PG in the sample or standards binds to HA on the plate. D) The b-PG:HA complex binds to streptavidin-labelled peroxidase which catalyses the colour reaction.

2.15 A proteomics approach to the identification of GAG elongation enzymes or monosaccharide transporter proteins in vascular SMCs

We used proteomics to search for potential therapeutically regulatable GAG elongation enzymes or monosaccharide transporters in vascular SMCs using a kinase inhibitor. The identity of the kinase inhibitor (KI) is commercially sensitive to our laboratory but irrelevant to current studies where it was being used as a tool, which decreases GAG length, and it may or may not have the same mechanism of action as fenofibrate.

The one-dimensional (1-DE) and two-dimensional electrophoresis (2-DE) patterns of cellular protein in human SMCs treated with genistein and PDGF or genistein/PDGF and the kinase inhibitor, which is a potent inhibitor of GAG elongation, was used as an alternative approach to studying differential gene expression. Confluent cultures of human vascular SMCs on 90 mm plates were treated with PDGF/genistein (100 μmol/L and 50 ng/mL, respectively) or PDGF/genistein/kinase inhibitor (100 μmol/L, 50 ng/mL, and 1 μmol/L, respectively, for 24 h.

For the 1-DE separation, cellular protein was obtained as described under Section 2.4. Following determination of the protein concentration using the BCA Colorimetric Assay, 12.5 μg, 25 μg and 50 μg of each sample were separated on a 160×200×1.5 mm, 12.5% SDS-PAGE gel according to Laemmli (Laemmli, 1970). Protein molecular weight markers (Invitrogen) were run in a separate lane. Proteins were stained with 0.025% Coomassie Blue.

For the 2-DE separation, cells were washed with a buffer containing 140 mmol/L sorbitol and 10 mmol/L Tris-HCl, pH 7.0 and lysed in a solution containing 8 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 60 mmol/L dithiothreitol (DTT) and 2% immobilized pH gradient (IPG) buffer pH3-10. Cells were scraped from the dish and placed in a 1.5 mL centrifuge tube. The insoluble material was removed by centrifugation for 10 min at room temperature. The protein

content was determined using the PlusOne™ 2-D Quant (Amersham Biosciences, Uppsala, Sweden). Samples containing 1 mg protein were mixed with a rehydration solution containing 8 mol/L urea, 2% CHAPS, 2% IPG buffer, pH 3-10, 130 mmol/L DTT and a trace of bromophenol blue to a total volume of 450 μl. The mixture was evenly applied to a ceramic IPG strip holder. Pre-cast IPG dry strips pH3-10 (24 cm), were positioned over the mixture gel side down and overlayed with Dry Strip cover fluid (Amersham Biosciences). The strip holders were covered and placed on the Ettan IPGphor unit platform (Amersham Biosciences) to rehydrate and focus for 24 h. Isoelectric focusing (IEF) was conducted at low voltages (500-1000 V) during the first 2 h and then continued with a maximum setting of 8000 V to reach a total of 64 kVh. The temperature was maintained at 20°C. After IEF separation, the gel strips were equilibrated 2×15 min in a solution containing 50 mmol/L Tris-HCl pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, 1% DTT. The second equilibration solution contained 4% iodoacetamide instead of DTT. The equilibrated strips were positioned on the surface of precast 12.5% SDS polyacrylamide vertical slabs (Amersham Biosciences, 255×196×1 mm). A filter (5×5 mm) spotted with pre-stained molecular weight markers (Invitrogen) was positioned at the end of the strip and both were sealed with 0.5% agarose containing a trace amount of bromophenol blue. The 2-DE was performed in the Ettan DALTII separation unit (Amersham Biosciences) for 1 h at power of 5 W/gel and then switched to 17 W/gel to maintain the temperature at 25°C until the bromophenol blue frontier reached the bottom of the gels (4-5 h). After 2-DE, the gels were stained with a heated solution (50-90°C) of 0.025% Coomassie blue. Gels were scanned on a UMAX flat bed scanner (Amersham Biosciences) and images were analyzed using the ImageMaster 2D Elite Version 4.01 software (Amersham Biosciences). Protein spots of interest were excised and the gel pieces were completely destained in a centrifuge tube with 50 mmol/L ammonium bicarbonate/50% methanol. Gel

slices were equilibrated in 20 mmol/L ammonium bicarbonate for 20 min followed by the addition of 100% acetonitrile for 10 min and this process was repeated three times. Excess liquid was removed by speed vacuum and the gel slices were dissolved in 20 mmol/L ammonium bicarbonate containing 10 µg/mL trypsin (Promega, Madison, WI, USA) at 37°C, overnight (16 h). The digestion buffer was removed and saved. The gel pieces were extracted once with 100 µl of 75% trifluoroacetic acid (TFA) for 30 min followed by 100 µL of 50% TFA/50% acetonitrile for 30 min. The two extracts plus the first saved digest buffer were pooled and concentrated to 10-20 µL. The sample was applied to a mass spectrometer (Ettan MALDI ToP, Amersham Biosciences) using αcyano-4-hydroxy-cinnamic acid (Laser Biolabs) matrix mixed 1:1 with the sample and the raw data was used for a database search using MS-Fit (http://prospector.ucsf.edu).

2.16 Graphics and Statistical Analysis

All graphics were created using Fig.P version 2.98 (Biosoft, Cambridge, UK). All data are expressed as the mean ± standard error of the mean (SEM). In some experiments the data were normalized to control values to adjust for variation between control values of individual experiments. Experiments were analyzed by a 1-way analysis of variance (ANOVA) or where indicated, a Student's t-test. Results were significant at $P<0.05$ or as stated in each individual figure. Refer to Appendix 1 for typical analysis of the data using a 1-way ANOVA.

Chapter 3: Treatment of human vascular SMCs with the PPAR-α ligand, fenofibrate, modifies proteoglycan structure

3.1 Introduction to results for Chapter 3

Vascular smooth muscle cells synthesize proteoglycans which are macromolecules consisting of a core protein with one or more negatively charged glycosaminoglycan chains, covalently attached. These molecules are thought to have a role in retaining lipoprotein in blood vessels, an initial step in the atherogenic casca te (Williams & Tabas, 1995, 1998), as well as an involvement in other vascular functions (Wight, 1989). Atherogenic growth factors induce alterations in proteoglycans, by increasing the expression of core proteins (Schonherr et al., 1993; Schonherr et al., 1991), increasing the length of glycosaminoglycans (Little et al., 2002) and by changing the sulfation pattern (Schonherr et al., 1993). The composition of GAGs is also altered in the atherosclerotic lesion (Evanko et al., 1998; Fernandez et al., 2003; Shanahan et al., 1997). Such changes in GAG structure have been shown (Little et al., 2002) or suggested (Edwards & Wagner, 1988; Gigli et al., 1993; Wight, 1980) to perturb the binding to LDL. Pharmacological agents that reverse or inhibit atherogenic changes in vascular proteoglycans (Nigro et al., 2002; Vijayagopal & Subramaniam, 2001) may advance the development of therapies directed at the vessel wall.

PPAR-α ligands such as fibrates are currently in use as lipid-lowering agents in patients with or without type 2 diabetes. Recent clinical evidence has indicated that the level of coronary narrowing in type 2 diabetes patients using these pharmacological agents is significantly reduced compared to the placebo group (Diabetes Atherosclerosis Intervention Study Investigators, 2001). We hypothesized that these agents may have direct vascular actions including an effect to modify proteoglycans in a way that would contribute to a decrease in binding to LDL. In this chapter, the effect of fenofibrate and several other PPAR-α ligands on the secretion, structure and fine chemical composition of proteoglycans in vascular SMCs is presented.

3.2   Results of Chapter 3

3.2.1   Characterization of human vascular SMCs

To characterize the *in vitro* model to be used for the study of proteoglycans, immunocytochemical staining of cells with SMC specific and non-SMC markers was used. Human vascular SMCs were positive for smooth muscle α-actin, a cytoskeletal protein present in vascular SMCs (Figure 3-1). Endothelial nitric oxide synthase (eNOS) is specifically expressed in vascular endothelial cells but not vascular smooth muscle cells. Human vascular SMCs were negative for eNOS indicating that the cultures contained primarily smooth muscle cells (Figure 3-1). Vimentin, an intermediate filament protein (53 kDa) that forms a fibrous meshwork throughout the cytoplasm (Muir, 1992), stains positive in proliferating SMCs and negative in quiescent SMCs. Serum deprived human vascular SMCs showed minimal staining of vimentin indicating that they were in the quiescent state (Figure 3-1). Additionally, human vascular SMCs were negative for proliferating cell nuclear antigen (PCNA) indicating that there was little proliferative activity (Figure 3-1). Smooth muscle cells, when grown to confluence, demonstrate characteristic "hills and valleys", unlike endothelial cells, which form a "cobblestone/honeycomb" arrangement (Ross, 1971). Phase contrast microscopy of confluent SMCs showed "hills and valleys" (Figure 3-2). These data indicate that the *in vitro* model to be used for the analysis of proteoglycans, contains positively identified SMCs.

SM α-actin   eNOS

PCNA   Vimentin

Figure 3-1

Immunocytochemical characterization of human vascular SMCs. Serum deprived human vascular SMCs stain positively for smooth muscle (SM) α-actin, weakly for proliferating cell nuclear antigen (PCNA) and vimentin and negatively for endothelial nitric oxide synthase (eNOS).

Figure 3-2

Phase contrast microscopy of human vascular SMCs. Serum deprived human vascular SMCs form "hills and valleys" when observed using phase contrast microscopy.

3.2.2 Human vascular smooth muscle cells express PPAR-α protein

It has been previously reported that three lines of human aortic SMCs express PPAR-α protein (Staels *et al.*, 1998a). We wanted to confirm these findings in the human vascular SMCs used to study the effects of PPAR-α ligands on vascular proteoglycans. Protein from hepatocytes (HepG2 cells) was used as a positive control because these cells express high levels of PPAR-α where its primary role is to regulate the expression of genes involved in fatty acid metabolism (Kliewer *et al.*, 2001; Sterchele *et al.*, 1996). HepG2 cells showed high levels of a ≈55 kDa band which has been previously described to correspond to the PPAR-α protein (Lemberger *et al.*, 1996), indicating that the antibody was suitable for the detection of PPAR-α protein in human vascular SMCs (Figure 3-3). We observed that human vascular SMCs express PPAR-α protein albeit at a lower level than for HepG2 cells (Figure 3-3). Vascular SMCs treated with the PPAR-α ligand, fenofibrate (30 µmol/L) or the PPAR-γ ligand, troglitazone (10 µmol/L) did not alter the level of PPAR-α protein (Figure 3-3). These data indicate that human vascular SMCs derived from the internal mammary artery express PPAR-α protein.

MW (kDa)

IMA SMCs

HepG2 Con FFB Trog

60.7 <PPARα

Figure 3-3

Western blot analysis of PPAR-α protein expression in human vascular SMCs. Protein

from human vascular SMCs treated with 0.1% DMSO (Con), fenofibrate (30 μmol/L; FFB) or

troglitazone (10 μmol/L; Trog) and the human hepatoma cell line (HepG2) were separated on

a SDS-polyacrylamide gel and blotted onto a nitrocellulose filter. Immunoreactivity was

detected by incubation with anti-human PPAR-α serum. Bands of the same size ($\approx$55 kDa,

arrow) were detected in HepG2 cells and SMCs, corresponding to the PPAR-α protein

(Lemberger *et al.*, 1996). Each lane contained 50 μg of protein as determined using the BCA

colorimetric assay.

3.2.3 Total cellular protein synthesis and the synthesis of proteoglycan core proteins - the effect of PPAR-α ligands

The study of the response of vascular SMCs to pharmacological treatment requires the establishment of a balance between the concentration for the desired effect and the concentration that does not induce unwanted effects such as cellular apoptosis. The analysis of total cellular protein synthesis was used to determine the appropriate concentrations of the PPAR-α ligands to study proteoglycan synthesis in human vascular SMCs that would not interfere with normal cellular processes. As a practical convenience this can be performed simultaneously with the investigation of proteoglycan core protein synthesis, by assessing [^{35}S]-methionine/cysteine ([^{35}S]-met/cys) incorporation into proteoglycans, secreted into the culture medium. Human SMCs were treated with various fibrates/PPAR-α ligands and assessed for the incorporation of [^{35}S]-met/cys into cell-associated total proteins. The fibrates/PPAR-α ligands chosen for this analysis were fenofibrate, the fenofibrate active metabolite, fenofibric-acid, a related and clinically used fibrate, gemfibrozil and a potent PPAR-α ligand used as a research tool, WY-14,643. Treatment of vascular SMCs with fibrates was performed in the presence and absence of TGF-β1. The culture medium was analyzed to quantitate the core protein of secreted proteoglycans using the CPC precipitation assay.

Fenofibrate treatment of vascular SMCs was associated with a 23.9% and 16.1% increase in [^{35}S]-met/cys incorporation into total cellular protein at 10 μmol/L and 30 μmol/L, respectively (Figure 3-4A). Fenofibrate (50 μmol/L) treatment of human vascular SMCs did not alter the incorporation of [^{35}S]-met/cys into total cellular protein however, 100 μmol/L fenofibrate inhibited the incorporation of [^{35}S]-met/cys into total cellular protein by 57.5% (Figure 3-4A). Stimulation of vascular SMCs with TGF-β1 increased the incorporation of

$[^{35}S]$-met/cys into total cellular protein by 24.8% ($778.7\pm113.6\times10^3$ cpm/well, $P<0.001$) compared to untreated cells ($605.8\pm61.8\times10^3$ cpm/well, Figure 3-4A). In contrast to basal conditions, fenofibrate treatment at 10 µmol/L or 30 µmol/L in the presence of TGF-β1 did not stimulate the incorporation of $[^{35}S]$-met/cys incorporation into total protein, compared to cells treated with TGF-β1 alone. However, fenofibrate treatment (100 µmol/L) in the presence of TGF-β1 decreased $[^{35}S]$-met/cys incorporation into total cellular protein by 64.5% ($P<0.001$) compared to cells treated with TGF-β1 alone (Figure 3-4A). The inhibition of $[^{35}S]$-met/cys incorporation into total cellular protein with fenofibrate (100 µmol/L) treatment under basal conditions and in the presence of TGF-β1 was taken as an indication of toxicity. In subsequent studies, the effects of fenofibrate on proteoglycans were studied at a concentration of up to 50 µmol/L.

Human vascular SMCs treated with fenofibrate (30 µmol/L) showed a significant increase (15.7%, $P<0.05$) in $[^{35}S]$-met/cys incorporation into total secreted proteoglycan core proteins compared to untreated cells however, this was not observed following treatment with 10 µmol/L or 50 µmol/L fenofibrate (Figure 3-4B). Treatment of SMCs with TGF-β1 (2 ng/mL) did not alter the $[^{35}S]$-met/cys incorporation into total secreted proteoglycan core protein compared to untreated cells (Figure 3-4B). Fenofibrate (10-50 µmol/L) treatment of human vascular SMCs in the presence of TGF-β1 showed no significant change in $[^{35}S]$-met/cys incorporation into total secreted core protein compared to cells treated with TGF-β1 alone (Figure 3-4B).

A

Total cellular protein synthesis

B

Total secreted proteoglycan core protein
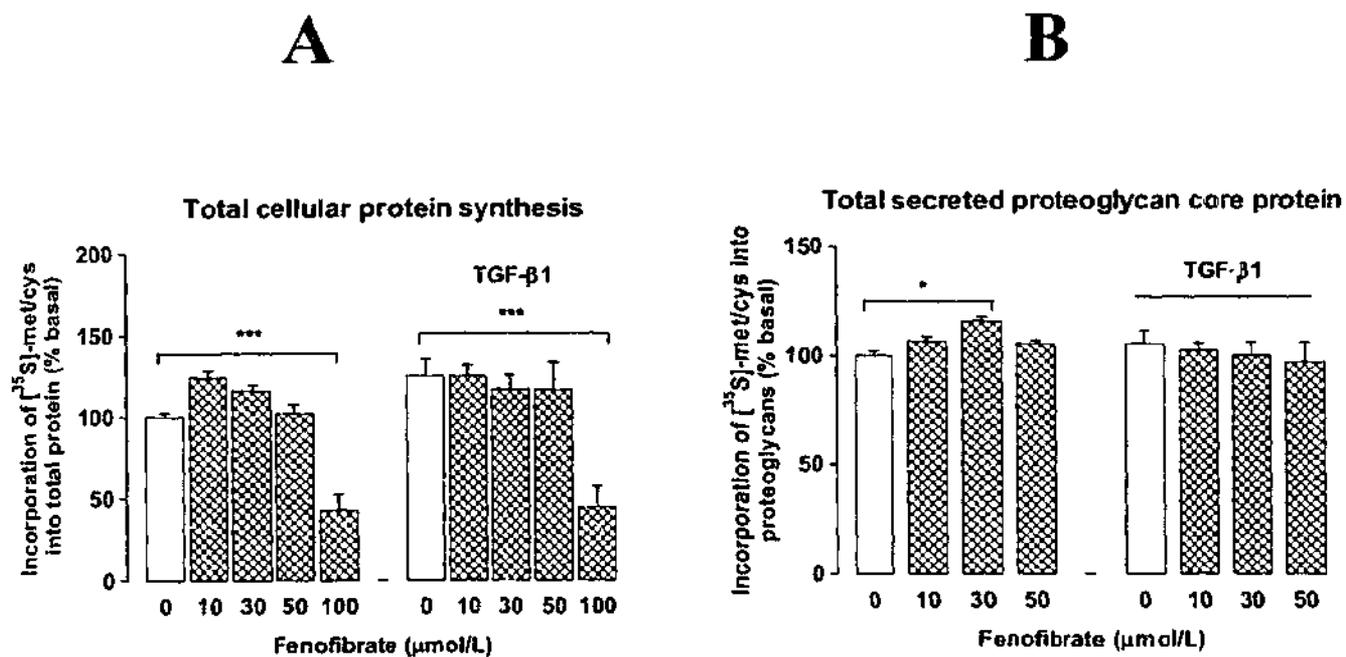
Figure 3-4

Effect of fenofibrate treatment on total cellular protein synthesis and secreted proteoglycan core protein synthesis in human vascular SMCs. Incorporation of [^{35}S]-met/cys into (A) total cellular protein or (B) secreted proteoglycan core protein following treatment of human vascular SMCs with fenofibrate (10-100 μmol/L or 10-50 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL). Data are normalized to each experimental control and presented as the mean±SEM of 3 experiments performed in triplicate (n=9, * $P<0.05$, ** $P<0.01$, ** $P<0.001$ using a 1-way ANOVA). Control value for total cellular protein is 605.8±61.8×10^3 cpm [^{35}S]-met/cys per well, control value for total proteoglycan core protein is 39.5±0.5×10^3 cpm [^{35}S]-met/cys per well.

The treatment of human vascular SMCs with fenofibric acid (100 μmol/L) increased [^{35}S]-met/cys incorporation into total cellular protein by 17.7% ($P<0.01$) compared to untreated cells (Figure 3-5A). Fenofibric acid (100 μmol/L) treatment in the presence of TGF-β1 did not alter [^{35}S]-met/cys incorporation into total cellular protein compared to cells treated with TGF-β1 alone (Figure 3-5A). Fenofibric acid treatment of vascular SMCs increased [^{35}S]-met/cys incorporation into total secreted proteoglycan core protein by 9.9% ($P<0.01$), compared to untreated cells (Figure 3-5B). In the presence of TGF-β1 and fenofibric-acid (100 μmol/L), vascular SMC proteoglycan core protein synthesis tended to decrease compared to cells treated with TGF-β1 alone (Figure 3-5B).

Treatment of human SMCs with gemfibrozil (100 μmol/L), did not alter the incorporation of [^{35}S]-met/cys into total cellular protein compared to cells maintained under basal conditions (Figure 3-6A). Gemfibrozil (100 μmol/L) treatment of vascular SMCs in the presence of TGF-β1 did not alter [^{35}S]-met/cys incorporation into total cellular protein compared to cells treated with TGF-β1 alone (Figure 3-6A). Gemfibrozil (100 μmol/L) treatment of SMCs significantly increased [^{35}S]-met/cys incorporation into total secreted proteoglycan core protein by 16.3% ($P<0.05$, Figure 3-6B). Human vascular SMCs treated with gemfibrozil (100 μmol/L) in the presence of TGF-β1 tended to decrease [^{35}S]-met/cys incorporation into total secreted proteoglycan core protein compared to cells treated with TGF-β1 alone (Figure 3-6B).

Human vascular SMCs treated with the potent PPAR-α ligand, WY-14,643 (100 μmol/L), showed no change in total protein synthesis compared to untreated cells, nor in the presence of TGF-β1 compared to cells treated with TGF-β1 alone (Figure 3-7A). Vascular SMCs treatment with WY-14,643 (100 μmol/L) did not alter [^{35}S]-met/cys incorporation into total

secreted proteoglycan core protein compared to cells maintained under basal conditions (Figure 3-7B). The treatment of vascular SMCs with WY-14,643 (100 µmol/L) in the presence of TGF-β1 did not alter [^{35}S]-met/cys incorporation into total secreted proteoglycan core protein synthesis compared to cells treated with TGF-β1 alone (Figure 3-7B).

Simultaneous analysis of total cellular protein and total secreted proteoglycan core protein synthesis allows for the interesting calculation of the contribution of proteoglycan core protein to total cellular protein synthesis. Assuming that the precursor pools of methionine/cysteine are similar and in equilibrium and that the specific activity of the pools and resulting protein are identical, we found that total cellular protein was approximately $605.8\pm61.8\times10^3$ cpm/well and total secreted proteoglycan core protein was $39.5\pm0.5\times10^3$ cpm/well. Thus, proteoglycan core protein contributes $\approx6.5\%$ of the total protein biosynthesis in human vascular SMCs.

In summary, these data indicate that the appropriate concentrations to study the effects of fibrates/PPAR-α ligands on vascular proteoglycans are 10-50 µmol/L for fenofibrate, and 100 µmol/L for fenofibric acid, gemfibrozil and WY-14,643. All fibrate related compounds increased vascular proteoglycan core protein synthesis however, the potent PPAR-α ligand, WY-14,643 did not have this effect. Under growth factor conditions, the fibrate related agents tended to reduce total proteoglycan core protein synthesis while WY-14,643 had no effect. Additionally, we have shown that proteoglycan synthesis is an appreciable fraction of the total protein synthesis. This indicates the importance and likely turnover rate of these macromolecules (the balance between proteoglycan degradation/synthesis) which occurs in the steady state vessel situation.

A

Total cellular protein synthesis

B

Total secreted proteoglycan core protein
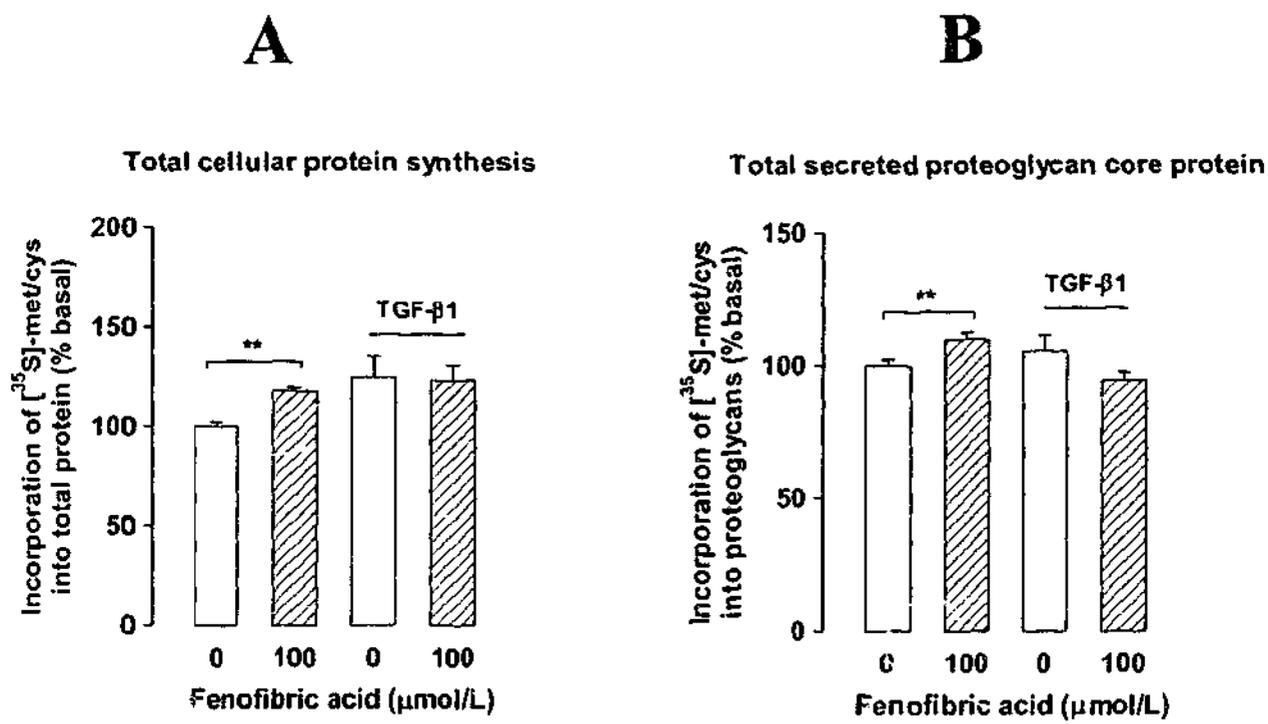
Figure 3-5

Effect of fenofibric acid treatment on total cellular protein synthesis and secreted proteoglycan core protein synthesis in human vascular SMCs. (A) Incorporation of $[^{35}S]$-met/cys into total cellular protein or (B) into secreted proteoglycan core protein following treatment of human vascular SMCs with fenofibric acid (100 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL). Data are normalized to each experimental control and presented as the mean±SEM of 3 experiments performed in triplicate (n=9, ** $P<0.01$ using a Student's t-test). Control value for total cellular protein is $605.8\pm61.8\times10^3$ cpm $[^{35}S]$-met/cys per well; control value for total proteoglycan core protein is $39.5\pm0.5\times10^3$ cpm $[^{35}S]$-met/cys per well.

A

Total cellular protein synthesis

B

Total secreted proteoglycan core protein
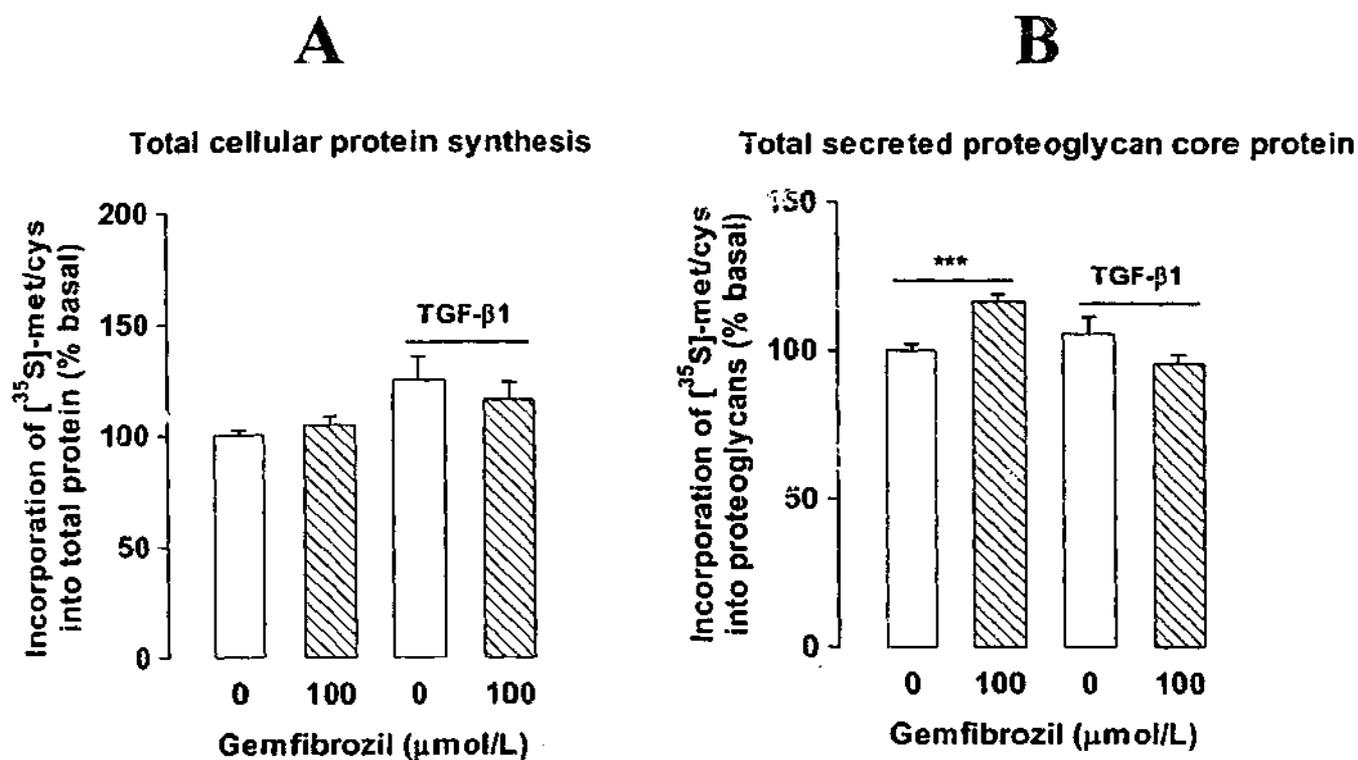
Figure 3-6

Effect of gemfibrozil treatment on total cellular protein synthesis and secreted proteoglycan core protein synthesis in human vascular SMCs. (A) Incorporation of [35S]-met/cys into total cellular protein or (B) into secreted proteoglycan core protein following treatment of human vascular SMCs with gemfibrozil (100 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL). Data are normalized to each experimental control and presented as the mean±SEM of 3 experiments performed in triplicate (n=9, *** $P<0.001$ using a 1-way ANOVA). Control value for total cellular protein is $605.8\pm61.8\times10^3$ cpm [35S]-met/cys per well; control value for total proteoglycan core protein is $39.5\pm0.5\times10^3$ cpm [35S]-met/cys per well.

A

Total cellular protein synthesis

B

Total secreted proteoglycan core protein
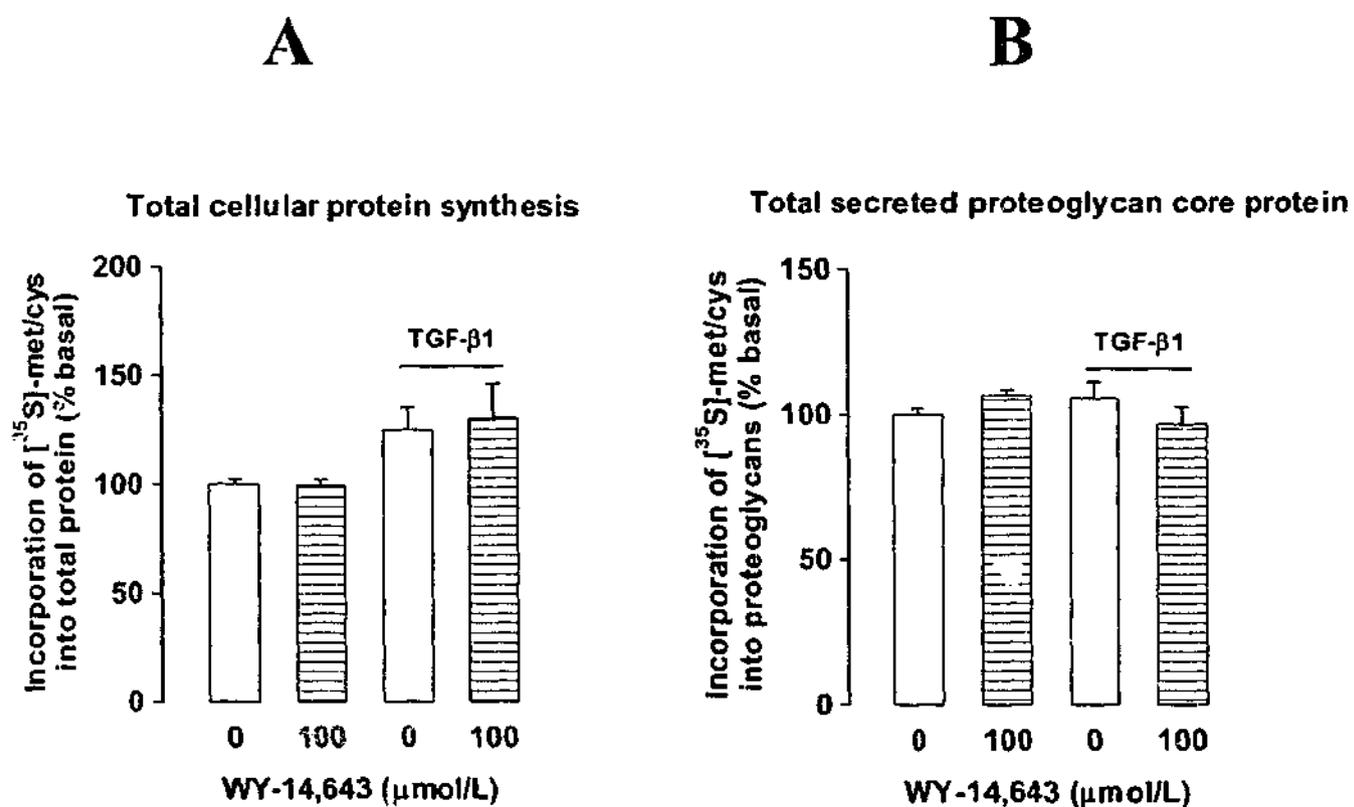
Figure 3-7

Effect of WY-14,643 treatment on total cellular protein synthesis and secreted proteoglycan core protein synthesis in human vascular SMCs. (A) Incorporation of $[^{35}S]$-met/cys into total cellular protein or (B) into secreted proteoglycan core protein following treatment of human vascular SMCs with WY-14,643 (100 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL). Data are normalized to each experimental control and presented as the mean±SEM of 3 experiments performed in triplicate (n=9). Control value for total cellular protein is $605.8 \pm 61.8 \times 10^3$ cpm $[^{35}S]$-met/cys per well; control value for total proteoglycan core protein is $39.5 \pm 0.5 \times 10^3$ cpm $[^{35}S]$-met/cys per well.

3.2.4 Analysis of core proteins following chondroitinase ABC digestion of GAGs – the effect of fenofibrate

The quantitation of proteoglycan core proteins by [^{35}S]-met/cys labelling and the CPC precipitation assay is a specific measure of total core protein synthesis. To assess individual core proteins the GAG chains can be enzymatically digested and the metabolically labelled core proteins separated by SDS-PAGE. Monkey (*Macaca nemestrina*) SMCs derived from the thoracic aorta were used because the proteoglycans produced by these cells have been studied in detail (Chang *et al.*, 1983; Schonherr *et al.*, 1993; Schonherr *et al.*, 1991) and they were the predominant cell line used by the laboratory of Professor Tom Wight at the Hope Heart Institute, Seattle, USA. Monkey SMCs were metabolically labelled with [^{35}S]-met/cys following treatment with fenofibrate (30 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL). Chondroitin sulfate/dermatan sulfate GAGs were digested with chondroitinase ABC and the proteoglycan core proteins were separated on a 4-13% linear gradient SDS-PAGE. Samples were loaded by equal aliquot so that the density of the core protein bands represents the amount of core protein in the sample. Computer image analysis was performed to provide data on the quantity and expression of the proteoglycan core proteins within each treatment.

The proteoglycan core protein bands were identified as versican, ($\approx$300 kDa), biglycan (43 kDa) and decorin (40 kDa, Figure 3-8A) by comparison to the work of Wight and colleagues (unpublished findings). Fenofibrate treatment of monkey SMCs showed that the pixel density of the versican core protein band was similar to that from cells maintained under basal conditions (Figure 3-8A and B). Monkey SMCs treated with fenofibrate showed a small reduction in the pixel density of biglycan and decorin core proteins compared to untreated cells (Figure 3-8A and B). Treatment of SMCs with TGF-β1 increased biglycan and decorin

core protein compared to untreated cells, as measured by an increase in the pixel density (Figure 3-8A and B). In the presence of TGF-β1, fenofibrate treatment of vascular SMCs reduced the synthesis of versican, biglycan and decorin core proteins compared to TGF-β1 alone, observed by a reduction in the density of these bands (Figure 3-8A and B).

The quantitation of total proteoglycan by the CPC precipitation assay, presented in Section 3.2.3, suggested that fenofibrate treatment increased total core protein synthesis (Figure 3-4). To assess whether fenofibrate treatment caused a general increase in the proteoglycan core proteins or specifically altered one of the proteoglycan core proteins, the expression of proteoglycan core proteins was assessed by analyzing the percentage pixel area of each core protein on SDS-PAGE (Figure 3-8C). Monkey SMCs express 44% versican, 33.6% biglycan and 22.4% decorin core proteins. Treatment of monkey vascular SMCs with fenofibrate showed a small increase in the expression of versican core protein from 44.0% to 47.1%, but no change in the expression of biglycan and decorin core proteins, compared to untreated cells (Figure 3-8C). Vascular SMCs treated with TGF-β1 showed a reduction in the expression of versican from 44.0% to 34.8% and an increase in the expression of biglycan core protein from 33.6% to 41.0% compared to untreated cells (Figure 3-8C). Fenofibrate treatment of vascular SMCs in the presence of TGF-β1 did not change the expression of versican, biglycan or decorin core proteins compared to cells treated with TGF-β1 alone (Figure 3-8C).
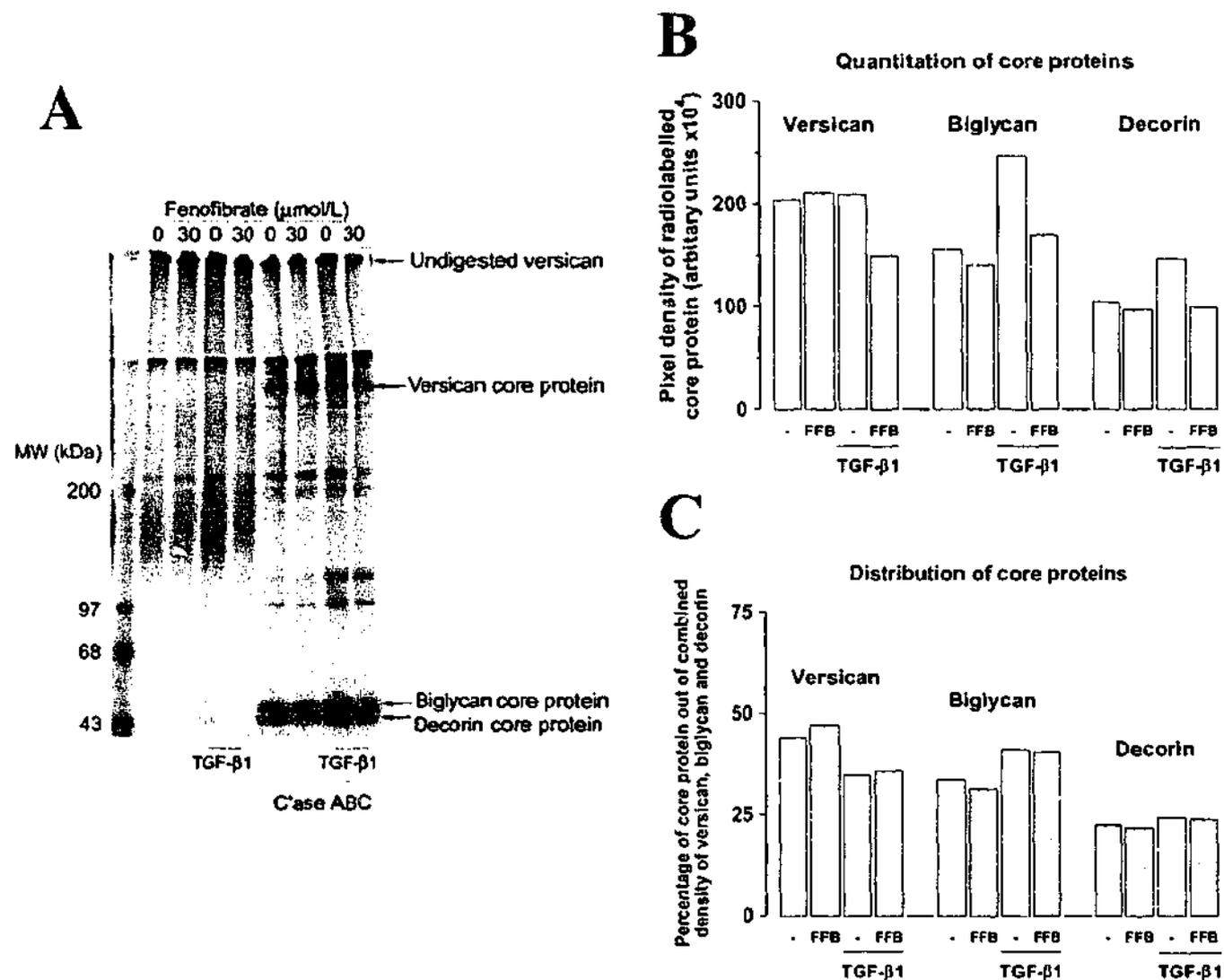
Figure 3-8

Effect of fenofibrate on vascular proteoglycan core protein synthesis and distribution.

Monkey thoracic aorta SMCs were treated with 0.1% DMSO (-) or fenofibrate (30 µmol/L; FFB) in the presence and absence of TGF-β1 (2 ng/mL) and metabolically labelled with [^{35}S]-met/cys. Samples were prepared by equal volume so that the density of the bands represents the amount of core protein in the sample. (A) Intact proteoglycans and proteoglycans digested with chondroitinase (C'ase) ABC were separated on a 4-13% linear gradient SDS-PAGE. (B) The pixel density of versican, biglycan and decorin core proteins and (C) the percentage of versican, biglycan and decorin core proteins within each lane on the gel calculated from the total density of versican+biglycan+decorin following C'ase ABC treatment. Analysis was performed using Image Quant 5.1. Gel represents 1 experiment.

In summary, these data show that fenofibrate treatment of vascular SMCs may specifically increase the expression of versican. These data complement the observations presented in Section 3.2.3, where fenofibrate treatment of vascular SMCs increased $[^{35}S]$-met/cys incorporation into total secreted proteoglycan core protein. In the presence of TGF-$\beta1$, fenofibrate treatment of vascular SMCs did not alter the expression of versican, biglycan and decorin core proteins. These data support those presented in Section 3.2.3 where fenofibrate treatment of vascular SMCs in the presence of TGF-$\beta1$, did not alter $[^{35}S]$-met/cys incorporation into total secreted proteoglycan core protein.

3.2.5 Quantitation of proteoglycan synthesis by $[^{35}S]$-sulfate incorporation - fenofibrate and fenofibric acid concentration-response

The complex structure of proteoglycans, which includes a core protein with one or more covalently attached sulfated and carboxylated glycosaminoglycan (GAG) chains, allows for multiple radio-chemical measures of synthesis. Core protein synthesis is measured by metabolically labelling with amino acids (e.g. $[^{35}S]$-met/cys) and the GAG chains may be labelled with $[^{35}S]$-sulfate, $[^{3}H]$-acetate, $[^{3}H]$-glucosamine or $[^{14}C]$-glucosamine. The effect of fenofibrate on vascular SMC proteoglycan core protein synthesis has been shown by measuring the incorporation of $[^{35}S]$-met/cys. To obtain a complete understanding of the effects of fibrate/PPAR-α ligand treatment on human vascular SMC proteoglycans, GAG chain synthesis was assessed by $[^{35}S]$-sulfate incorporation. Confluent smooth muscle cells were treated with fenofibrate (0.3-50 µmol/L) or fenofibric acid (0-100 µmol/L) in the presence and absence of atherogenic growth factors, TGF-$\beta1$ (1 ng/mL) and PDGF (50 ng/mL) and metabolically labelled with $[^{35}S]$-sulfate. The incorporation of $[^{35}S]$-sulfate into secreted proteoglycans was quantitated using the CPC precipitation assay.

Fenofibrate (50 μmol/L) treatment of SMCs inhibited [^{35}S]-sulfate incorporation into vascular proteoglycans by 21.8% ($P<0.05$) compared to untreated cells (Figure 3-9A). Stimulation of human vascular SMCs with TGF-β1 (1 ng/mL), increased [^{35}S]-sulfate incorporation into vascular proteoglycans by 26.8% ($P<0.01$) from 285.4±12.6 cpm/10^3 cells to 355.6±13.3 cpm/10^3 cells confirming the work of Little *et al.* (Little *et al.*, 2002). In the presence of TGF-β1, fenofibrate (50 μmol/L) treatment of SMCs decreased [^{35}S]-sulfate incorporation into vascular proteoglycans by 28.6% ($P<0.01$) compared to cells treated with TGF-β1 alone (Figure 3-9B). Stimulation of vascular SMCs with PDGF increased proteoglycan synthesis by 62.5% from 118.4±4.4 cpm/10^3 cells to 190.9±10.5 cpm/10^3 cells ($P<0.001$) compared to untreated cells. In the presence of PDGF, treatment of vascular SMCs with fenofibrate (30 μmol/L and 50 μmol/L) reduced [^{35}S]-sulfate incorporation into proteoglycans by 30.7% ($P<0.01$) and 51.8% ($P<0.001$), respectively compared to cells treated with PDGF alone (Figure 3-9C).
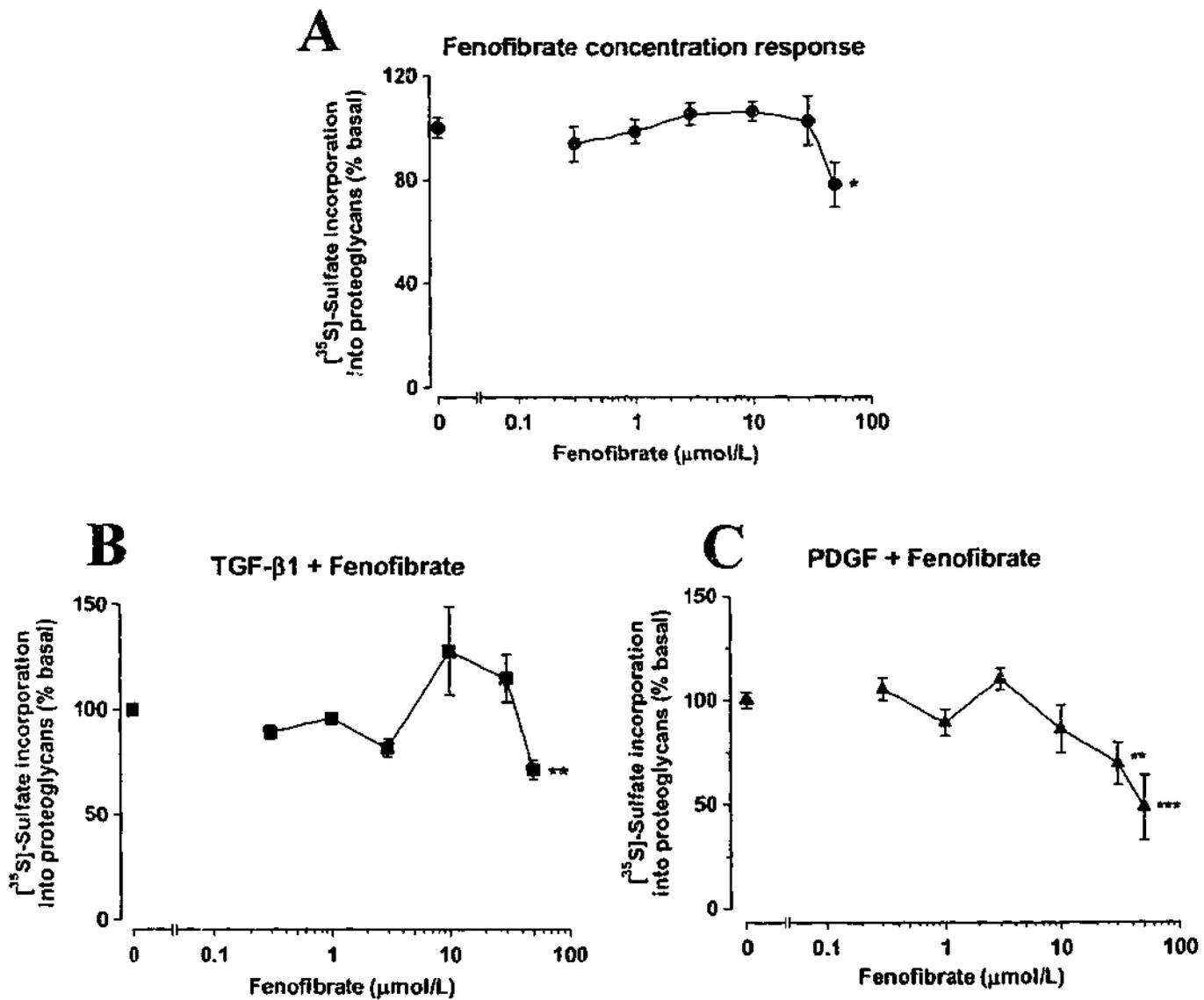
Figure 3-9

Treatment of vascular SMCs with fenofibrate decreases [^{35}S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with (A) fenofibrate (0.3-100 μmol/L) alone or in the presence of (B) TGF-β1 (1 ng/mL) or (C) PDGF (50 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Proteoglycans secreted into the culture medium were quantitated using the CPC precipitation assay. Parallel plates were prepared to assess cell number. Data was expressed as cpm/10^3 cells and then normalized to the control (285.4±12.6 cpm [^{35}S]-sulfate/10^3 cells for A, 355.6±13.3 cpm [^{35}S]-sulfate/10^3 cells for B, 190.9±10.5 cpm [^{35}S]-sulfate/10^3 cells for C). Values are mean±SEM of 2 experiments performed in triplicate (n=6, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ using a 1-way ANOVA).

Vascular SMCs treated with fenofibric acid (100 μmol/L and 300 μmol/L) showed a 14.6% ($P<0.05$) and 17.1% ($P<0.05$) decrease in [^{35}S]-sulfate incorporation into proteoglycans, respectively (Figure 3-10A). In the presence of TGF-β1, fenofibric acid (100 μmol/L and 300 μmol/L) reduced [^{35}S]-sulfate incorporation into proteoglycans by more than 30% ($P<0.001$), compared to cells treated with TGF-β1 alone (Figure 3-10B). The treatment of human SMCs with fenofibric acid (1-300 μmol/L) in the presence of PDGF showed a sharp reduction in [^{35}S]-sulfate incorporation into proteoglycans at the beginning of the concentration-response curve (Figure 3-10C). Treatment of vascular SMCs with fenofibric acid (100 μmol/L and 300 μmol/L) in the presence of PDGF decreased [^{35}S]-sulfate incorporation into proteoglycans by 27.6% and 43.6% ($P<0.05$), respectively (Figure 3-10C). The number of cells at the end of the treatment period with 300 μmol/L fenofibric acid ($65.6\pm4.0\times10^4$ cells/well) was significantly different from cells maintained under basal conditions ($77.4\pm1.7\times10^4$ cells/well, $P<0.01$). These data suggest that the effect of fenofibric acid (300 μmol/L) to reduce [^{35}S]-sulfate incorporation into proteoglycans may be associated with the reduced cell number.

In summary these data show that the treatment of vascular SMCs with both fenofibrate (0.3-50 μmol/L) fenofibric acid (1-300 μmol/L) causes small but consistent inhibition of proteoglycan synthesis in the presence and absence of growth factors, TGF-β1 and PDGF. If the assumption was made that the bottom of the concentration-response curves was the maximum response, the approximate IC_{50} value for fenofibrate would be 3-10 μmol/L. If the same assumptions were applied to the data observed with fenofibric acid, the approximate IC_{50} value for fenofibric acid would be 30 μmol/L. Hence, the relative potency of the two compounds to induce effects on proteoglycan synthesis, may not be directly related to their

PPAR-α activity but may be linked to the cell permeability, which is determined by the chemical structure of the compounds. The basic chemical structure of fenofibrate is an ester that is hydrophobic and thus cell permeable however, it is not a direct PPAR-α ligand and requires chemical cleavage by cellular esterases to release the active acid derivative (fenofibric acid). Cell permeability of fenofibric acid would be lower than that for fenofibrate however it is the active ligand for PPAR-α. The hypothesis that cell permeability of fibrates rather than PPAR-α activity may perturb proteoglycan synthesis is addressed in Section 3.2.14.

Despite the 24 h period required to assess proteoglycan biosynthesis, these experiments looked at the acute effects of fenofibrate applied at the time of cell stimulation. As fibrates may act as PPAR-α ligands and thus modify gene transcription we later looked at the effects of pre-incubation on the inhibitory effects of fenofibrate (see Section 3.2.12).
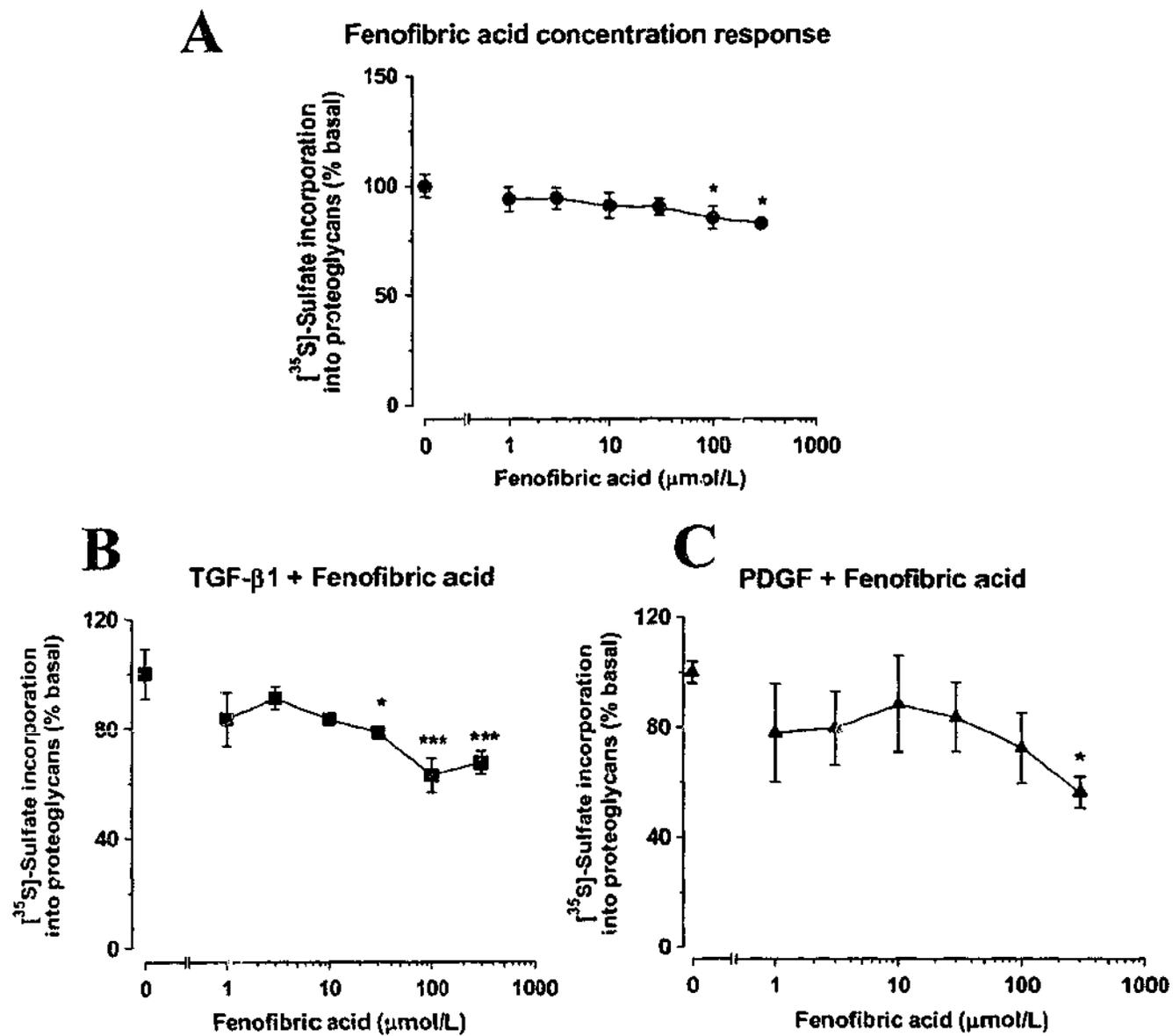
Figure 3-10

Treatment of vascular SMCs with fenofibric acid decreases [^{35}S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with (A) fenofibric acid (1-300 µmol/L) alone or in the presence of (B) TGF-β1 (1 ng/mL) or (C) PDGF (50 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Proteoglycans secreted into the culture medium were quantitated using the CPC precipitation assay. Parallel plates were prepared to assess cell number. Data was expressed as cpm/10^3 cells and then normalized to the control (176.1±23.3 cpm [^{35}S]-sulfate/10^3 cells for A, 223.8±36.1 cpm [^{35}S]-sulfate/10^3 cells for B, 190.9±10.5 cpm [^{35}S]-sulfate/10^3 cells for C). Values are mean±SEM of 2 experiments performed in triplicate (n=6, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ using a 1-way ANOVA).

3.2.6 Quantitation of proteoglycan synthesis by [^{3}H]-glucosamine incorporation – the effect of fenofibrate

From the assessment of [^{35}S]-sulfate incorporation into GAGs, it would be expected that similar results would be obtained by [^{3}H]-glucosamine labelling. However, glucosamine is subject to completely different metabolism from sulfate and assessment of radio-labelling with both constituents is the preferred option for a full and complete understanding of the effects of the fibrates/PPAR-α ligands on proteoglycan biosynthesis. Vascular SMCs were metabolically labelled with [^{3}H]-glucosamine rather than [^{14}C]-glucosamine, partially because the former is less expensive. To extend the observations that fenofibrate treatment of vascular SMCs leads to a reduction in [^{35}S]-sulfate incorporation (Section 3.2.5), vascular SMCs were metabolically labelled with [^{3}H]-glucosamine. Vascular SMCs were treated with fenofibrate (50 µmol/L), as this was the concentration which gave the greatest effect to reduce [^{35}S]-sulfate incorporation (see Section 3.2.5). The effect of fenofibrate treatment on human vascular SMCs was assessed in the presence and absence of TGF-β1 and PDGF. The results are expressed as [^{3}H]-hexosamine incorporation to reflect both [^{3}H]-galactosamine within CS/DS GAGs and [^{3}H]-glucosamine within HS GAGs/hyaluronan which may also be precipitated by the CPC assay.

As predicted from earlier work (Section 3.2.5), vascular SMCs treated with the growth factors, TGF-β1 and PDGF, increased [^{3}H]-hexosamine incorporation into GAGs 2.5-fold ($P<0.01$) and 3-fold ($P<0.001$), respectively compared to untreated cells (Figure 3-11). The treatment of vascular SMCs with fenofibrate (50 µmol/L) increased [^{3}H]-hexosamine incorporation into GAGs by 95.6% ($P<0.05$) (Figure 3-11). Treatment of vascular SMCs with fenofibrate (50 µmol/L) in the presence of TGF-β1, further stimulated the incorporation of [^{3}H]-hexosamine into GAGs, compared to cells treated with TGF-β1 alone (Figure 3-11).

Similarly, the treatment of vascular SMCs with fenofibrate (50 μmol/L) in the presence of PDGF, increased [^{3}H]-hexosamine incorporation into GAGs by 32.6% ($P<0.05$) compared to cells treated with PDGF alone (Figure 3-11). These data are in contrast to the effect of fenofibrate treatment on vascular SMCs to reduce [^{35}S]-sulfate incorporation into secreted proteoglycans in the presence and absence of TGF-β1 and PDGF (Section 3.2.5). These anomalous results and the question raised by them were investigated in detail and are presented in Chapter 5.
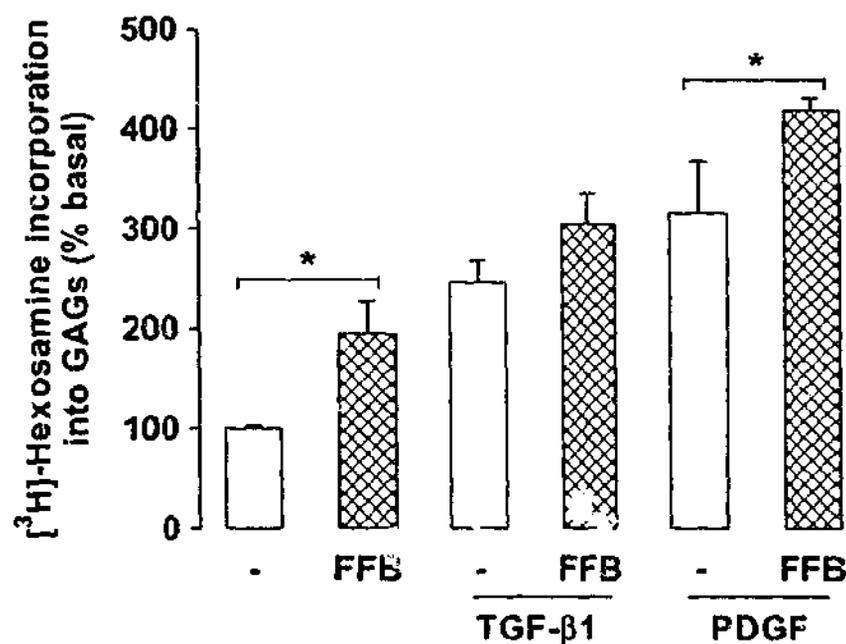
Figure 3-11

Vascular SMCs treated with fenofibrate show increased [³H]-hexosamine incorporation into GAGs. Human vascular SMCs were treated with 0.1% DMSO (-) or fenofibrate (FFB, 50 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) or PDGF (50 ng/mL) and metabolically labelled with [³H]-glucosamine. Glycosaminoglycans were precipitated by the CPC assay. Data represents the mean±SEM of 2 experiments performed in triplicate (* $P<0.05$ using a 1-way ANOVA). Data was expressed as cpm/10^3 cells and then normalized to the basal value (164.4±37.3 cpm [³H]-hexosamine/10^3 cells). These data should be contrasted with the inhibitory effects of fenofibrate on [³⁵S]-sulfate incorporation into proteoglycans shown in Figure 3-9.

3.2.7 Analysis of electrophoretic mobility of proteoglycans by SDS-PAGE – the effect of fenofibrate and fenofibric acid

The treatment of vascular SMCs with fenofibrate and fenofibric acid in the presence of atherogenic growth factors, showed a reduction in $[^{35}S]$-sulfate incorporation into proteoglycans (Section 3.2.5). The reduction in sulfate incorporation may be associated with a reduction in GAG length. Glycosaminoglycans are the site of lipoprotein binding and retention (Williams & Tabas, 1995, 1998) and the length of the GAGs has been shown to influence binding to LDL. For example, vascular proteoglycans synthesized in the presence of atherogenic growth factors (e.g. TGF-β1) show an increase in GAG length which increases the binding to LDL (Little *et al.*, 2002), while shorter GAG chains such as those synthesized in the presence of glucosamine (Tannock *et al.*, 2002a) or calcium channel antagonists (Vijayagopal & Subramaniam, 2001), show reduced LDL binding. To determine whether or not the reduction in $[^{35}S]$-sulfate incorporation into proteoglycans following the treatment of vascular SMCs with fenofibrate or fenofibric acid, was associated with a decrease in GAG length, radiolabelled proteoglycans were sized by SDS-PAGE. Proteoglycans separated by SDS-PAGE appear as broad bands due to heterogeneity of GAG chain length. In these experiments the SDS-PAGE data was further analyzed by computer analysis to give an indication of the migration of the bands and the range of the molecular mass of the proteoglycans.

Imaging of the gels showed five bands (Figure 3-12 to 3-17): 1) a tight band which remains in the stacking gel and has been previously characterized as versican (Schonherr *et al.*, 1991; Yao *et al.*, 1994), 2) a band which enters the resolving gel but does not migrate contains predominantly HS GAGs (see Section 3.2.11) and may be perlecan, 3) a large band >300 kDa which has not been identified but contains HS/CS GAG chains (see Section 3.2.11), 4) a band

with an apparent molecular mass of ≈200 kDa and has been previously identified as biglycan (Jarvelainen *et al.*, 1991) and was digested with chondroitinase ABC (see Section 3.2.11) and 5) a band of apparent molecular mass ≈100 kDa which has been previously characterized as decorin (Jarvelainen *et al.*, 1991) and was digested with chondroitinase ABC (see Section 3.2.11).

Treatment of human vascular SMCs with fenofibrate (0.3-50 μmol/L) produced proteoglycans which showed a concentration-related increase in the electrophoretic mobility by SDS-PAGE (Figure 3-12A and B). Stimulation of vascular SMCs with either TGF-β1 or PDGF which increase GAG length (Little *et al.*, 2002; Schonherr *et al.*, 1993; Schonherr *et al.*, 1991), retards the migration of proteoglycans by SDS-PAGE, compared to proteoglycans from cells maintained under basal conditions (Figure 3-13A and B and 3-14A and B). In the presence of TGF-β1, treatment of SMCs with fenofibrate (0.3-50 μmol/L) increased the electrophoretic mobility of bands corresponding to biglycan and decorin, in a concentration-related manner (Figure 3-13A and B). Bands corresponding to biglycan and decorin synthesized by vascular SMCs treated with fenofibrate (0.3-50 μmol/L) in the presence of PDGF, show a concentration-related increase in the electrophoretic mobility by SDS-PAGE (Figure 3-14A and B). These data suggest that the maximum effect of fenofibrate treatment, in the presence and absence of growth factors, to increase the electrophoretic mobility of proteoglycans is 30 μmol/L indicating that the IC_{50} value is 3-10 μmol/L for inhibiting GAG elongation. These data show that the changes in GAG length occur on both biglycan and decorin suggesting that changes in GAG synthesis may not be dependent on the identity of the core protein and also validates the use of xyloside to study changes in GAG synthesis (see section 3.2.8).
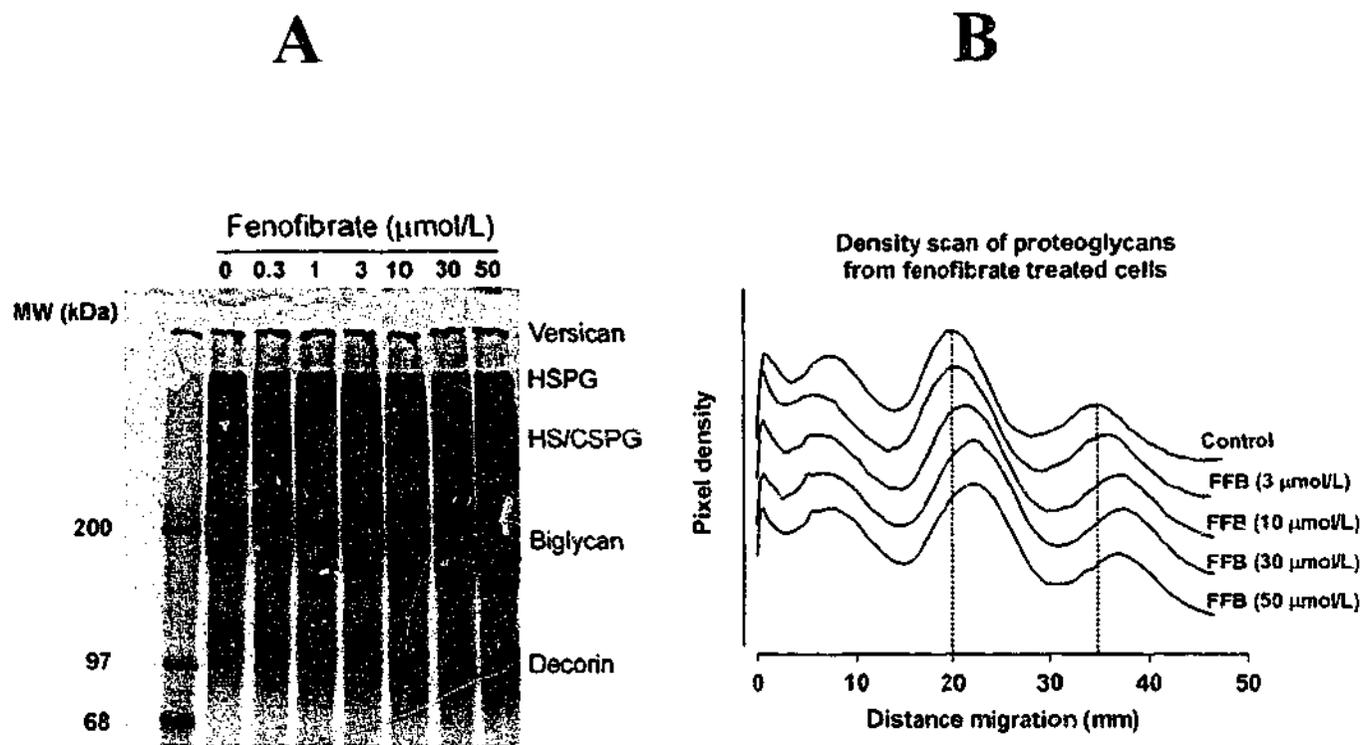
Figure 3-12

Treatment of vascular SMCs with fenofibrate produces proteoglycans with increased electrophoretic mobility. Vascular SMCs were treated with fenofibrate (0.3-50 μmol/L) and metabolically labelled with $[^{35}S]$-sulfate. (A) Secreted proteoglycans were isolated and separated on a 4-13% separating gel with 3.5% stacking gel and radiolabelled molecular weight standards were loaded in a separate lane. Each lane contained 20×10^3 cpm. (B) Graphical representation of the migration of radiolabelled proteoglycans in the resolving gel from control (0.1% DMSO) and fenofibrate (FFB; 0.3-50 μmol/L) treated SMCs from the gel presented in A. The dotted lines are the peak migration of biglycan and decorin from untreated SMCs. To facilitate data analysis, scans were strategically positioned to reflect the position on the gel using Fig.P Version 2.98. Gel represents 2 experiments with similar results.

A

TGF-β1 +
Fenofibrate (µmol/L)
0 0.3 1 3 10 30 50 Con

Versican
HSPG
HS/CSPG

MW (kDa)

200   Biglycan

97   Decorin

68

B

Density scan of proteoglycans
from TGF-β1 + fenofibrate treated SMCs

Pixel density

Control
TGF-β1
FFB (0.3 µmol/L)
FFB (3 µmol/L)
FFB (30 µmol/L)

0   10   20   30   40   50
Distance migration (mm)
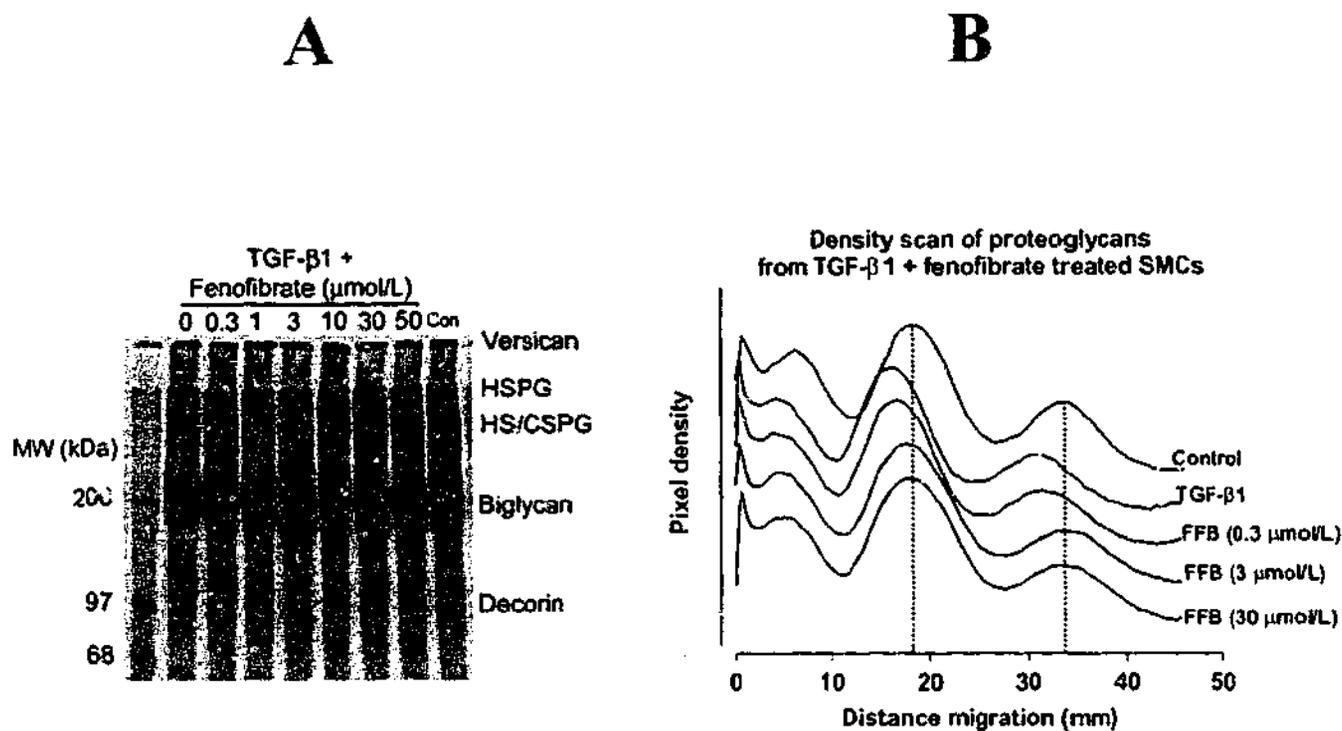
Figure 3-13

Treatment of vascular SMCs with fenofibrate in the presence of TGF-β1 produces proteoglycans with increased electrophoretic mobility. Human vascular SMCs were treated with fenofibrate (0.3-50 µmol/L) in the presence of TGF-β1 (1 ng/mL) and metabolically labelled with $[^{35}S]$-sulfate. (A) Secreted proteoglycans were isolated and separated on a 4-13% separating gel with 3.5% stacking gel. Each lane contained 20×10^3 cpm and radiolabelled molecular weight standards were loaded in a separate lane. Con is 0.1% DMSO without TGF-β1. (B) Graphical representation of the migration of radiolabelled proteoglycans from control, TGF-β1 and fenofibrate (FFB; 0.3-50 µmol/L) with TGF-β1 treated SMCs from the gel presented in A. The dotted lines are the peak migration of biglycan and decorin from untreated SMCs. To facilitate data analysis, scans were strategically positioned to reflect the position on the gel using Fig.P Version 2.98. Gel represents 2 experiments with similar results.

A

PDGF +
Fenofibrate (µmol/L)

Con   0   0.3   1   3   10   30   50

Versican
HSPG
MW (kDa)
HS/CSPG
250
Biglycan
Decorin
98

B

Density scan of proteoglycans
from PDGF + fenofibrate treated SMCs

Pixel density

Control
PDGF
FFB (30 µmol/L)
+PDGF

0   10   20   30

Distance migration (mm)
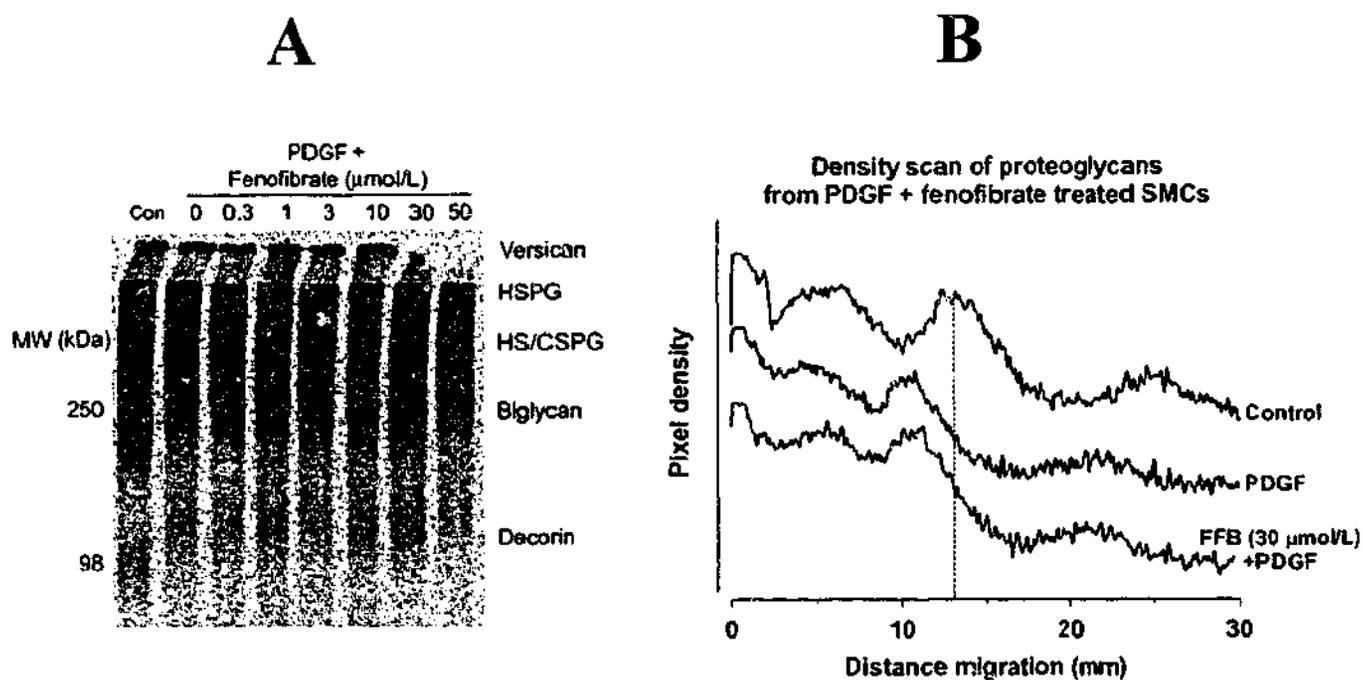
Figure 3-14

Treatment of vascular SMCs with fenofibrate in the presence of PDGF produces proteoglycans with increased electrophoretic mobility. Human vascular SMCs were treated with fenofibrate (0.3-50 µmol/L) in the presence of PDGF (50 ng/mL) and metabolically labelled with $[^{35}S]$-sulfate. (A) Secreted proteoglycans were isolated and separated on a 4-13% separating gel with 3.5% stacking gel. Each lane contained 20×10^3 cpm. Non-radiolabelled molecular weight protein markers were run in a separate lane. Con is 0.1% DMSO without TGF-β1. (B) Graphical representation of the migration of radiolabelled proteoglycans from control, PDGF and fenofibrate (30 µmol/L; FFB) with PDGF treated SMCs from the gel presented in A. Dotted line is the peak migration of the biglycan from untreated SMCs. To facilitate data analysis, scans were strategically positioned to reflect the position on the gel using Fig.P Version 2.98. Gel represents 2 experiments with similar results.

Vascular proteoglycans synthesized in the presence of fenofibric acid (1-300 µmol/L) showed a concentration-related increase in electrophoretic mobility by SDS-PAGE, with a maximum effect observed at 100 µmol/L (Figure 3-15A and B). The electrophoretic mobility of proteoglycans synthesized by SMCs in the presence of fenofibric acid (1-300 µmol/L) and TGF-β1 was increased in a concentration-related manner, with a maximum effect observed at 100 µmol/L (Figure 3-16A and B). In the presence of PDGF, fenofibric acid (1-300 µmol/L) treatment of vascular SMCs generated proteoglycans that showed a concentration-related increase in electrophoretic mobility (Figure 3-17A and B). The data show that the maximum effect of fenofibric acid treatment, in the presence and absence of growth factors, to increase the electrophoretic mobility of proteoglycans is 100 µmol/L suggesting that the IC_{50} value is 10 µmol/L.

A

Fenofibric acid (µmol/L)

0 1 3 10 30 100 300

MW (kDa)

250

98

64

Versican
HSPG
HS/CSPG
Biglycan
Decorin

B

Density scan of proteoglycans from
fenofibric acid treated SMCs

Pixel density

Distance migration (mm)

Control
F-acid (3 µmol/L)
F-acid (30 µmol/L)
F-acid (300 µmol/L)
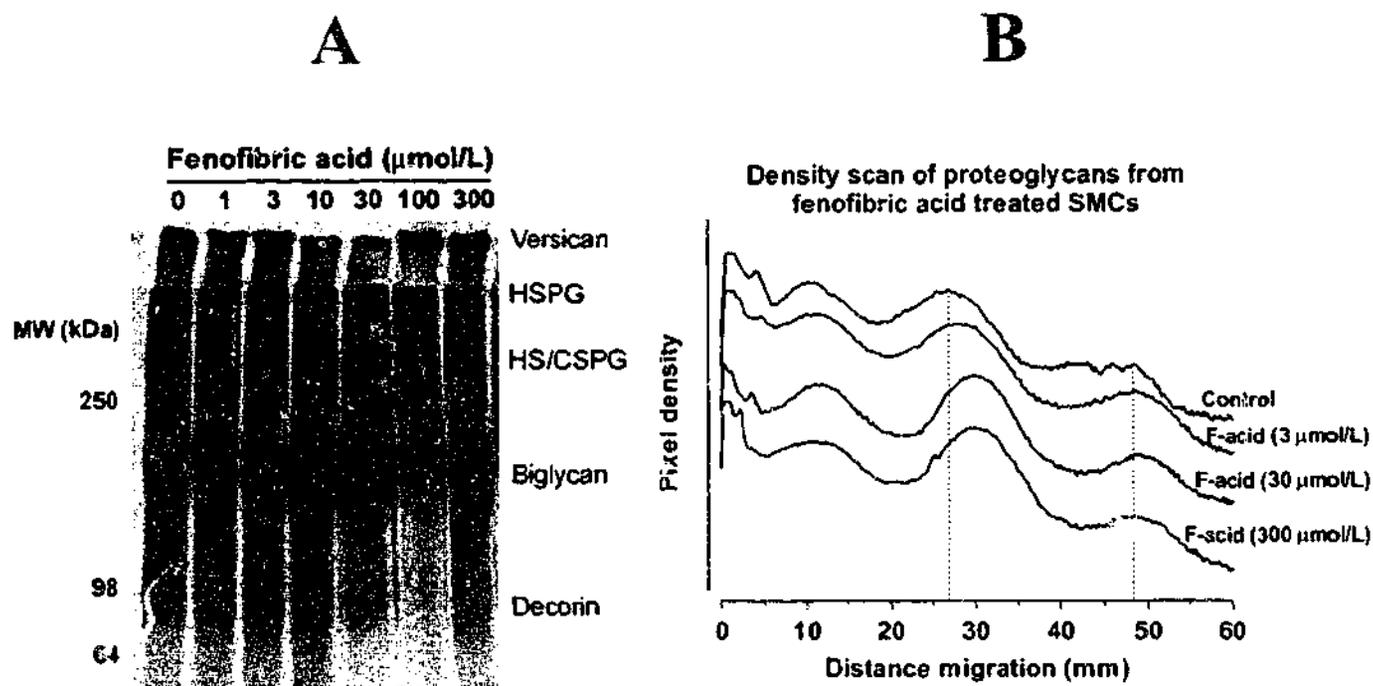
Figure 3-15

**Human vascular SMCs treated with fenofibric acid synthesize proteoglycans with
increased electrophoretic migration by SDS-PAGE.** Human vascular SMCs were treated

with fenofibric acid (1-300 µmol/L) and metabolically labelled with [^{35}S]-sulfate. (A) Secreted

proteoglycans were isolated and separated on a 4-13% separating gel with 3.5% stacking gel.

Non-radiolabelled molecular weight protein markers were run in a separate lane. (B)

Graphical representation of the migration of radiolabelled proteoglycans from control (0.1%

DMSO) and fenofibric acid (3-300 µmol/L; F-acid) treated SMCs from the gel presented in A.

Dotted lines are the peak migration of biglycan and decorin from untreated SMCs. To

facilitate data analysis, scans were strategically positioned to reflect position on the gel using

Fig.P Version 2.98. Gel represents 2 experiments with similar results.

A

TGF-β1 +
Fenofibric acid (μmol/L)

0 1 3 10 30 100 300 Con

Versican
HSPG

MW (kDa)

HS/CSPG

250

Biglycan

98

Decorin

B

Density scan of proteoglycans from
TGF-β1 + fenofibric acid treated SMCs

Control
TGF-β1
F-acid (3 μmol/L)
F-acid (30 μmol/L)
F-acid (300 μmol/L)

Pixel density

Distance migration (mm)
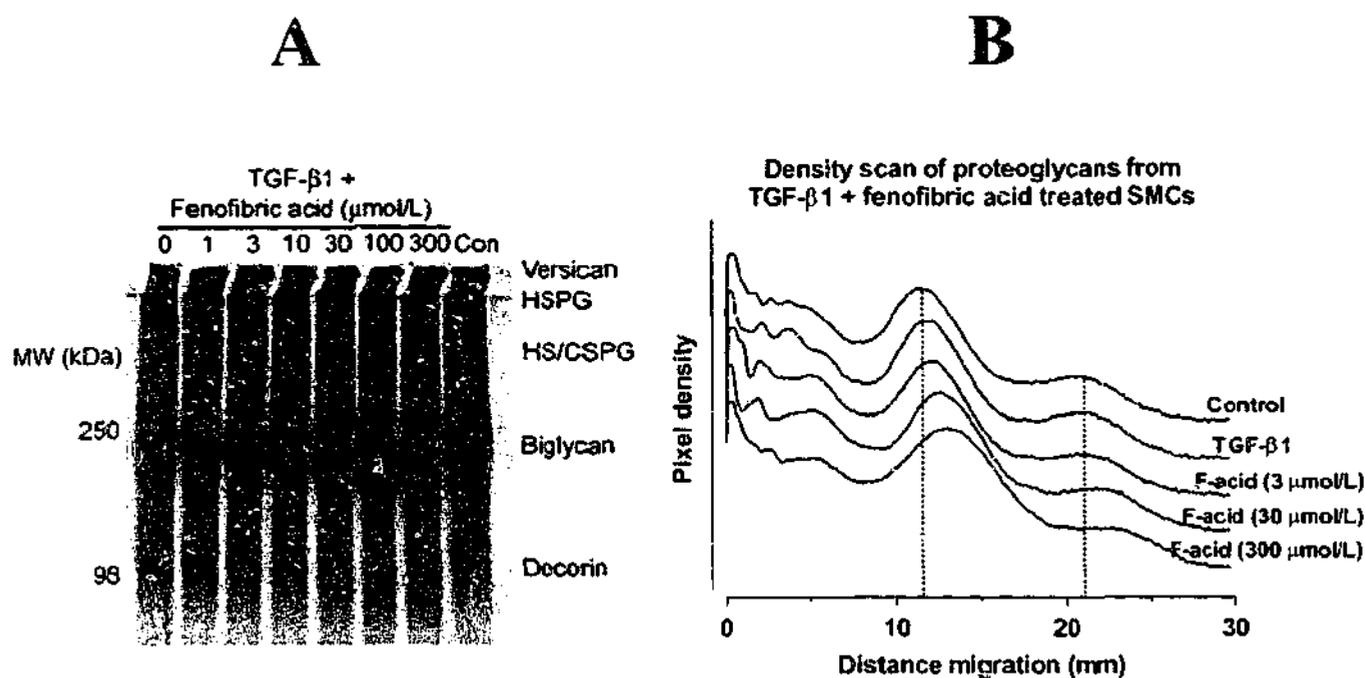
Figure 3-16

Human vascular SMCs treated with fenofibric acid in the presence of TGF-β1 synthesize proteoglycans with increased electrophoretic migration by SDS-PAGE. Human vascular SMCs were treated with fenofibric acid (1-300 μmol/L) in the presence of TGF-β1 (1 ng/mL) and metabolically labelled with [^{35}S]-sulfate. (A) Secreted proteoglycans were isolated and separated on a 4-13% separating gel with 3.5% stacking gel. Each lane contained 20×10^3 cpm. Non-radiolabelled molecular weight protein markers were run in separate lane. Con is 0.1% DMSO without TGF-β1. (B) Graphical representation of the migration of radiolabelled proteoglycans from control, TGF-β1 and fenofibric acid (3-300 μmol/L; F-acid) with TGF-β1 treated SMCs from the gel presented in A. Dotted lines are the peak migration of biglycan and decorin from untreated SMCs. To facilitate data analysis, scans were strategically positioned to reflect position on the gel using Fig.P Version 2.98. Gel represents 2 experiments with similar results.

A

PDGF +
Fenofibric acid (μmol/L)

Con 0 1 3 10 30 100 300

Versican
HSPG
HS/CSPG
Biglycan
Decorin

MW (kDa)
250
98

B

Density scan of proteoglycans from
PDGF + fenofibric acid treated SMCs

Pixel density

Control
PDGF
F-acid (100 μmol/L) +PDGF

0   10   20   30
Distance migration (mm)

Figure 3-17

Human vascular SMCs treated with fenofibric acid in the presence of PDGF synthesize

proteoglycans with increased electrophoretic migration by SDS-PAGE. Human vascular

SMCs were treated with fenofibric acid (1-300 μmol/L) in the presence of PDGF (50 ng/mL)

and metabolically labelled with $[^{35}S]$-sulfate. (A) Secreted proteoglycans were isolated and

separated on a 4-13% separating gel with 3.5% stacking gel. Each lane contained 20×10^3 cpm.

Non-radiolabelled molecular weight protein markers were run in a separate lane. Con is 0.1%

DMSO without TGF-β1. (B) Graphical representation of the migration of radiolabelled

proteoglycans from control, PDGF and fenofibric acid (100 μmol/L; F-acid) with PDGF

treated SMCs from the gel presented in A. Dotted line is the peak migration of biglycan from

untreated SMCs. To facilitate data analysis, scans were strategically positioned to reflect

position on the gel using Fig.P Version 2.98. Gel represents 2 experiments with similar results.

In summary, these data show that fenofibrate and fenofibric acid treatment of vascular SMCs increases the electrophoretic mobility of secreted proteoglycans in the presence and absence of atherogenic growth factors, TGF-β1 and PDGF. The increase in electrophoretic mobility of proteoglycans following the treatment of vascular SMCs with either fenofibrate or fenofibric acid was taken as an indication of a reduction in GAG chain length. The separation of proteoglycans by SDS-PAGE is predominantly dependent upon molecular size however, the method is also based on charge allowing for the possibility that a change in the charge density of the molecules may also play a role in the migration by SDS-PAGE. The effect of fenofibrate and fenofibric-acid to increase the electrophoretic mobility of proteoglycans suggests that there is a concentration-related decrease in GAG length and/or an increase in the negative charge of the proteoglycans. We therefore assessed the size of the proteoglycans synthesized by vascular SMCs treated with fenofibrate, using size exclusion chromatography (Section 3.2.9) and the charge of the proteoglycans was analyzed using fluorophore assisted carbohydrate electrophoresis (FACE, Section 3.2.10) and by analytical DEAE (see Chapter 4, Section 4.5.2).

3.2.8 Analysis of GAG synthesis using media supplementation with exogenous xyloside – the effect of fenofibrate

Xylose is the initial sugar moiety of the linkage region involved in the initiation of GAG biosynthesis. In the presence of exogenous xyloside, SMCs secrete a mixture of proteoglycans and free xyloside-initiated GAGs (secreted free chains). Exogenous xyloside acts as a false acceptor for GAG chain synthesis and thus represents an independent assay of GAG biosynthesis (refer to Chapter 1, Figure 1-13) (Little *et al.*, 2002; Potter-Perigo *et al.*, 1992). To test the hypothesis that fenofibrate treatment of vascular SMCs inhibits GAG

elongation processes, xyloside was supplemented into the culture medium of vascular SMCs followed by treatment with fenofibrate in the presence and absence of TGF-β1 and metabolic labelling with [^{35}S]-sulfate. The short GAG chains initiated and synthesized on xyloside and then secreted will be referred to as "secreted free chains". Incorporation of [^{35}S]-sulfate into proteoglycans and secreted free chains was assessed by the CPC precipitation assay and the electrophoretic migration of secreted free chains was assessed by SDS-PAGE.

Incorporation of [^{35}S]-sulfate into secreted free chains was reduced by 27.3% (P <0.05) in cells treated with fenofibrate (30 μmol/L) and TGF-β1 (2 ng/mL) compared to TGF-β1 alone (Figure 3-18A). Secreted free chains were separated from intact proteoglycans using SDS-PAGE and the electrophoretic migration was analyzed (Figure 3-18B and C). Treatment of vascular SMCs reduces the electrophoretic migration of the secreted free chains, indicating that there is an increase in the length of these chains (Figure 3-18B and C) as previously shown by Little *et al* (Little *et al.*, 2002). Following treatment with fenofibrate (30 μmol/L) the SMCs synthesized secreted free chains with an increased electrophoretic mobility compared to no drug, under both basal conditions and in the presence of TGF-β1 (Figure 3-18B and C). These data indicate that fenofibrate reduces the apparent length and/or charge of the secreted free chains by directly inhibiting the GAG elongation processes occurring most likely in the SMC Golgi apparatus. It also shows that the effect of drugs, in this case fenofibrate, can be directed at the GAG elongation processes, independent of the presence or function of proteoglycan core protein.
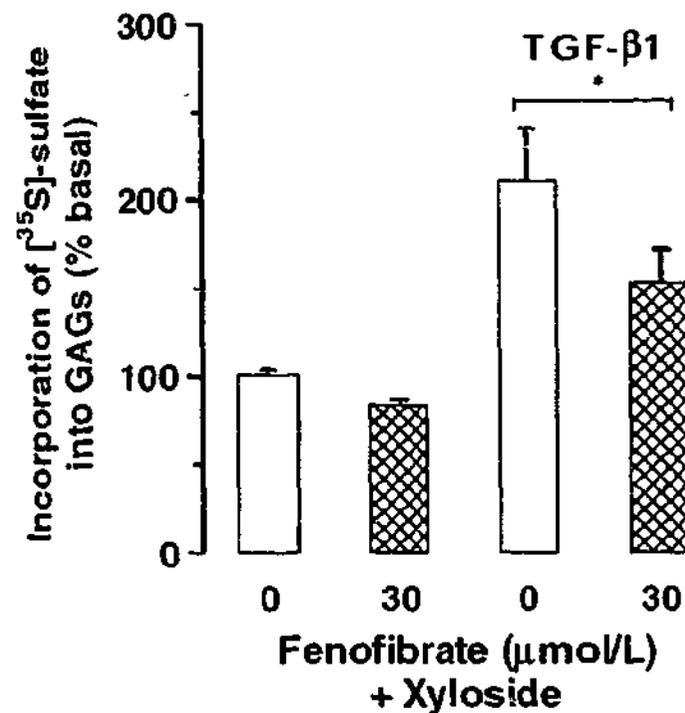
Figure 3-18

Treatment of vascular SMCs with fenofibrate and TGF-β1 decreases [35S]-sulfate incorporation into secreted free chains. Human vascular SMCs supplemented with xyloside (0.5 mmol/L) were treated with fenofibrate (30 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) and metabolically labelled with [35S]-sulfate. Incorporation of [35S]-sulfate into proteoglycan and secreted free chains was quantitated using the CPC precipitation assay. Each bar represents the mean±SEM from 2 separate experiments and was analyzed using a 1-way ANOVA (n=6, *P <0.05 TGF-β1 versus TGF-β1 plus fenofibrate).

A

Fenofibrate (μmol/L)

0 30 0 30 0 30 0 30

Verscian

MW (kDa)

HSPG
HS/CSPG

250

Biglycan

Decorin

98
64
50
36
30
16

Secreted free chains
"Xyloside-GAGs"

TGF   TGF

0.5 mmol/L
Xyloside

B

Density scan of secreted free chains
from fenofibrate treated SMCs

Pixel density

Control

FFB (30 μmol/L)

TGF-β1

FFB (30 μmol/L)
+TGF-β1

25   35   45

Distance migration (mm)
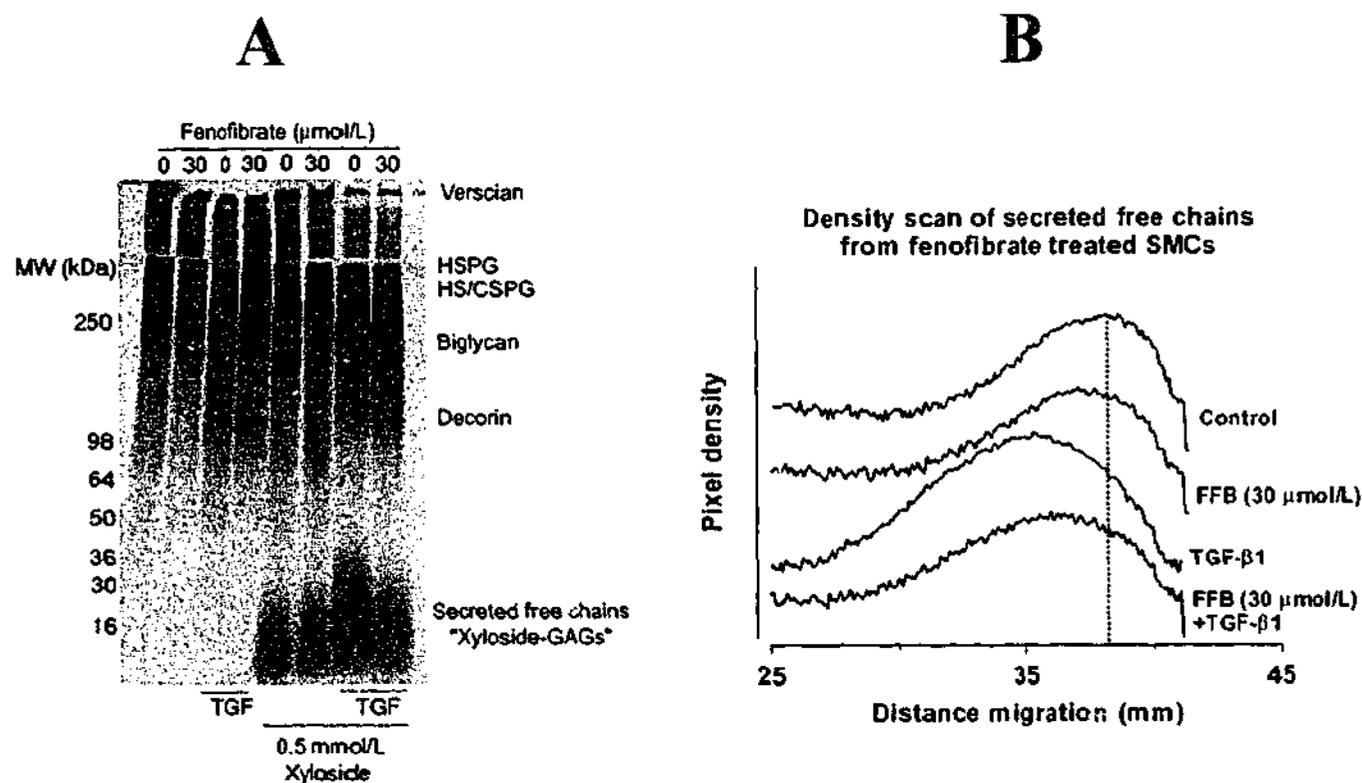
Figure 3-19

Treatment of vascular SMCs with fenofibrate and TGF-β1 increases the electrophoretic mobility of secreted free chains. Human vascular SMCs supplemented with xyloside were treated with fenofibrate (30 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Secreted proteoglycans and free chains were isolated from human vascular SMCs treated with xyloside supplemented medium or control medium and were treated with fenofibrate in the presence and absence of TGF-β1 (2 ng/mL). (A) Intact proteoglycans and secreted free chains were separated by SDS-PAGE. (B) The electrophoretic migration of the secreted free chains from A was graphically represented. Dotted line is the peak migration of xylo-GAGS from untreated SMCs. To facilitate data analysis, scans were strategically positioned to reflect position on the gel using Fig.P Version 2.98. Gel represents 2 experiments with similar results.

3.2.9 Analysis of proteoglycan molecular sizes by size exclusion chromatography – effect of fenofibrate

Analysis of GAG length by SDS-PAGE provides data on the smaller proteoglycans, biglycan and decorin but not the larger proteoglycans such as versican and perlecan (see section 3.2.7). Gel filtration chromatography assesses hydrodynamic size independent of any ionic charge effects which may contribute to analyses by SDS-PAGE. Gel filtration chromatography was used to complete the analysis of molecular size of all the secreted proteoglycans, cleaved GAG chains and xyloside-initiated GAGs following treatment of cells with fenofibrate (30 μmol/L). Intact proteoglycans were applied to a CL-2B column, while the smaller cleaved GAG chains and xyloside-initiated GAGs were applied to a Sepharose CL-6B column. Each fraction was assigned a K_{av} value to normalize the results from each column run (see Chapter 2, Section 2.8.1 for the calculation of K_{av}). It follows that larger molecules have smaller K_{av} values and smaller molecules have larger K_{av} values.

Monkey aortic SMCs produce versican with a K_{av} value of ≈0.30 while the K_{av} value of biglycan/decorin is ≈0.70 following sizing on a Sepharose CL-2B column (Tannock *et al.*, 2004). Intact proteoglycans from human vascular SMCs sized on a Sepharose CL-2B column eluted as one peak, with a peak K_{av} value of 0.74±0.02 (Figure 3-20A). These molecular size data indicate that human vascular SMCs produce predominantly biglycan/decorin. The chromatogram shows only little radioactivity at the K_{av} value of 0.3, indicating that human vascular SMCs produce small amounts of versican (Figure 3-20A and B). The chromatograms have a shoulder between K_{av} values 0.55 and 0.65 (Figure 3-20A and B) and this may correspond to the >300 kDa proteoglycan observed by SDS-PAGE (Figure 3-12).

Treatment of human vascular SMCs with fenofibrate (30 μmol/L) showed that there was no difference between the peak K_{av} value of intact proteoglycans (0.75±0.02) compared to

proteoglycans from untreated cells (Figure 3-20A and Table 3-1). Intact proteoglycans from cells stimulated with TGF-β1 have a smaller K_{av} value (0.72±0.02) than proteoglycans from cells maintained under basal conditions (Figure 3-20B and Table 3-1). Vascular SMCs treated with fenofibrate (30 μmol/L) in the presence of TGF-β1 showed no difference in the peak K_{av} value of intact proteoglycans compared to proteoglycans from cells treated with TGF-β1 alone (Figure 3-20B and Table 3-1).

Effects of fenofibrate treatment on GAG chains may be masked when analyzing complete proteoglycans due to the large contribution of the core protein when size is obviously unchanged. Therefore, free GAG chains were released following chemical cleavage of the intact [^{35}S]-sulfate labelled proteoglycans, using sodium borohydride β-elimination and were sized on a Sepharose CL-6B column (Figure 3-21A and B). The chains from proteoglycans synthesized under basal conditions had a peak K_{av} value of 0.470±0.006 while chains from cells treated with fenofibrate (30 μmol/L) had a peak K_{av} value of 0.467±0.003 (Figure 3-21A and Table 3-1). Vascular SMCs stimulated with TGF-β1 produced GAG chains with a peak K_{av} value of 0.417±0.003, while the chains from proteoglycans synthesized in the presence of fenofibrate and TGF-β1 had a peak K_{av} value of 0.430±0.006 (Figure 3-21B and Table 3-1). The data from Wasteson (Wasteson, 1971) included a range of low molecular weight chondroitin sulfate GAGs sized on a Sepharose CL-6B column and a mathematical relationship that could convert the K_{av} to molecular weight values. We used the data from Wasteson (Wasteson, 1971) to calculate the approximate molecular weight (see Chapter 2, Section 2.8.1 for the calculation of K_{av}) for the cleaved chains from human vascular SMCs (Table 3-1). The small changes in K_{av} observed following treatment of vascular SMCs with fenofibrate relate to a change in molecular weight of cleaved GAG chains from 32.0±0.8 kDa to 30.0±1.3 kDa (not significant) from TGF-β1 versus TGF-β1 with fenofibrate, respectively.

This represents a 6.3% decrease in molecular weight of fenofibrate treatment in the presence of TGF-β1, which corresponds to ≈ 8 monosaccharides on a GAG chain.

Secreted free chains were also sized on a Sepharose CL-6B column (Figure 3-22A and B). The treatment of xyloside supplemented vascular SMCs with fenofibrate lead to the synthesis of secreted free chains with a peak K_{av} value of 0.63 ± 0.02 which was not different from the secreted free chains synthesized under basal conditions (peak K_{av} value of 0.63 ± 0.01, Figure 3-22A and Table 3-1). Secreted free chains synthesized in the presence of TGF-β1 were larger than those synthesized without growth factor and eluted at K_{av} 0.59 ± 0.02 (Figure 3-21B and Table 3-1). Fenofibrate reduced the size of secreted free chains in the presence of TGF-β1 to a K_{av} value of 0.60 ± 0.03 compared to secreted free chains from cells treated with TGF-β1 alone (Figure 3-22B and Table 3-1). The calculated molecular weight for secreted chains decreased from 13.3 ± 1.5 kDa to 12.5 ± 1.6 kDa (not significant) in TGF-β1 vs TGF-β1 with fenofibrate treated SMCs, respectively (Table 3-1). This represents a 6.0% decrease in molecular weight of GAG following fenofibrate treatment in the presence of TGF-β1, which corresponds to ≈ 3-4 monosaccharides on a secreted free chain. Thus, human vascular SMCs treated with fenofibrate induces small but consistent reductions in the length of GAGs on vascular proteoglycans in the presence of the atherogenic growth factor, TGF-β1 and indicates that the inhibitory effect of fenofibrate is manifest directly on the GAG chain elongation mechanisms within the cell.
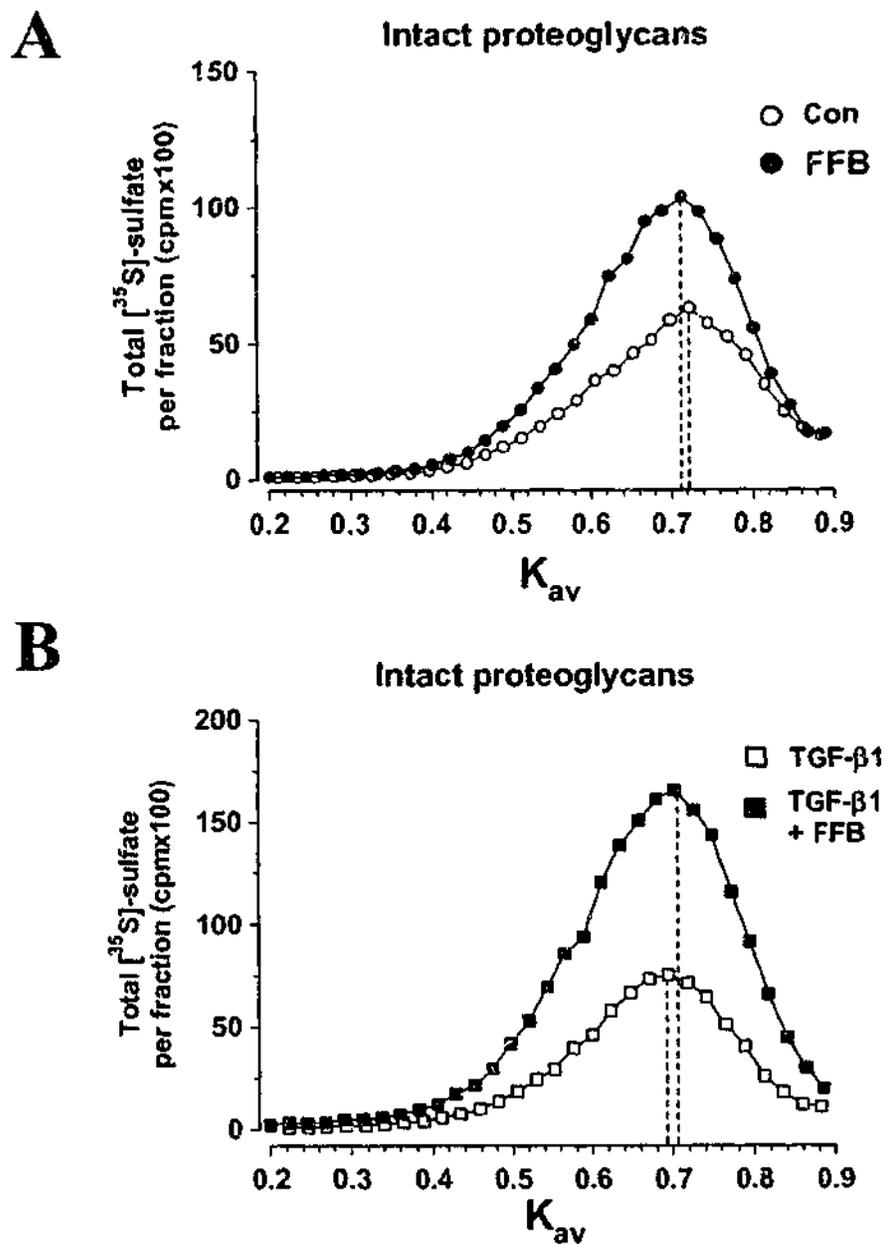
Figure 3-20

Analysis of the size of secreted <u>intact proteoglycans</u> synthesized in the presence of fenofibrate. (A) Proteoglycans from human SMCs treated with 0.1% DMSO (Con; open circles) or fenofibrate (FFB; 30 μmol/L; closed circles) sized on a Sepharose CL-2B column. (B) Proteoglycans from SMCs treated with TGF-β1 (2 ng/mL; open squares) and TGF-β1 with fenofibrate (30 μmol/L; closed squares) sized on Sepharose CL-2B column. Dashed lines indicate the peak of each chromatogram. Chromatograms represent 1 result from 3 different experiments with similar data.

A

Cleaved GAG chains

○ Con
● FFB

B

Cleaved GAG chains

□ TGF-β1
■ TGF-β1 + FFB
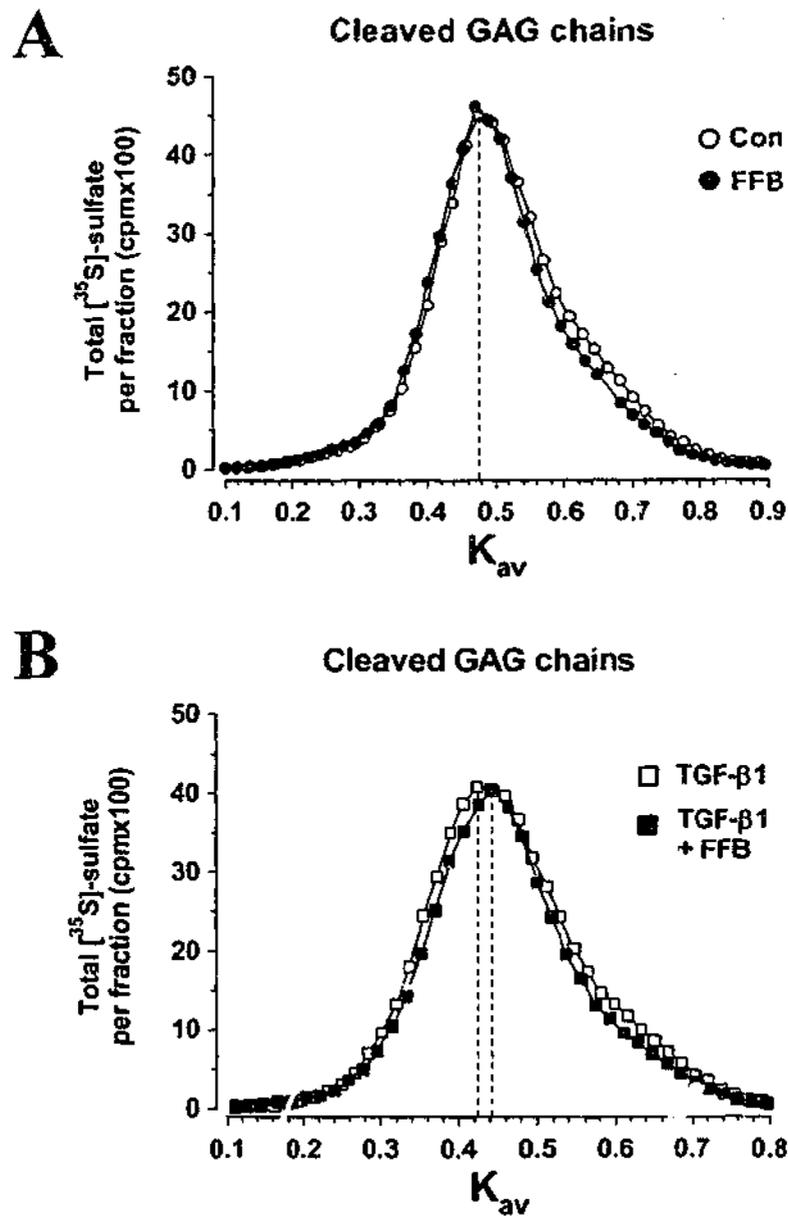
Figure 3-21

Analysis of the size of c*r*eaved GAG chains from proteoglycans synthesized in the presence of fenofibrate. A) GAG chains were cleaved from proteoglycans synthesized by human SMCs treated wi h 0.1% DMSO (Con; open circles) or fenofibrate (FFB; 30 μmol/L; closed circles) and siz*r* J on a Sepharose CL-6B column. (B) GAG chains were cleaved from proteoglycans synthe*s* zed by human SMCs treated with TGF-β1 (2 ng/mL; open squares) and TGF-β1 with fenofib *r*ate (30 μmol/L; closed squares) on Sepharose a CL-6B column. Dashed lines indicate the p *r*ak of each chromatogram. Chromatograms represent 1 result from 3 different experiment *s* with similar data.

A Secreted free chains

B Secreted free chains
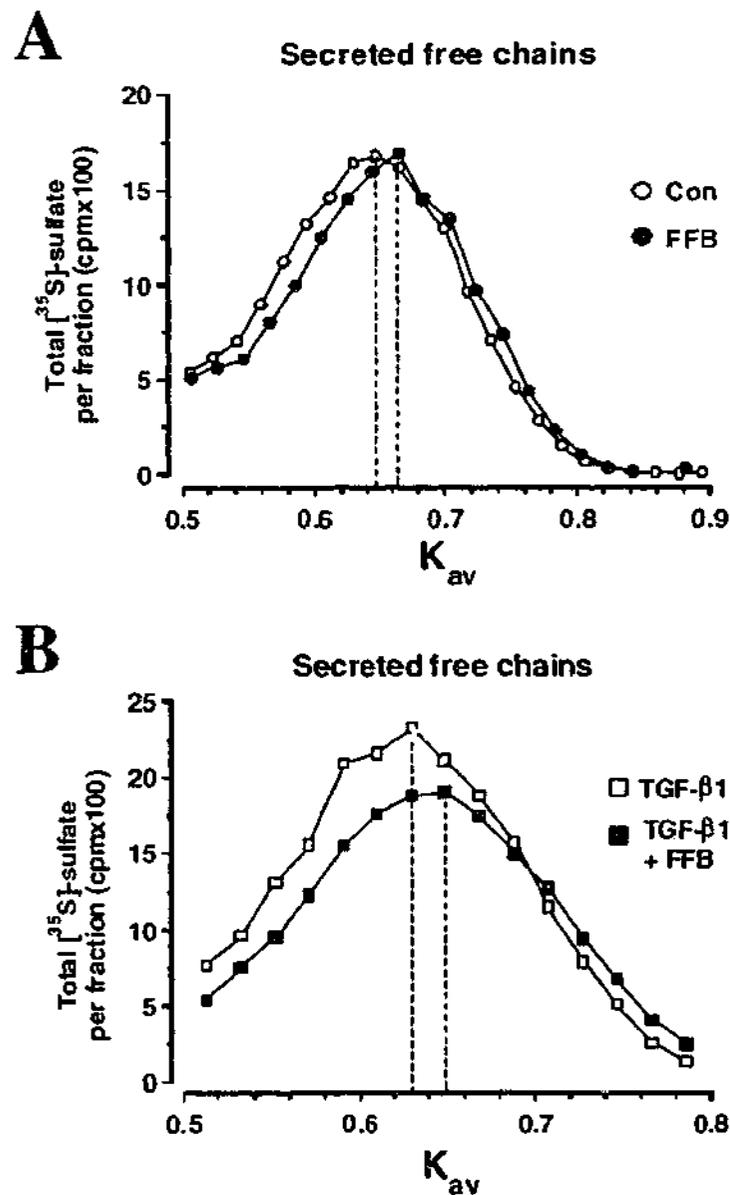
Figure 3-22

Size analysis of <u>secreted free chains</u> from xyloside supplemented human vascular SMCs treated with fenofibrate. (A) Secreted free chains from xyloside (0.5 mmol/L) supplemented human vascular SMCs in the presence of 0.1% DMSO (Con; open circles) or fenofibrate (FFB; 30 μmol/L; closed circles), were sized on a Sepharose CL-6B column. (B) Secreted free chains from human vascular SMCs treated with TGF-β1 (2 ng/mL; open squares) and TGF-β1 with fenofibrate (30 μmol/L; closed squares). Dashed lines indicate the peak of each chromatogram. Chromatograms represent 1 result from 3 different experiments with similar data.

	Control	Fenofibrate	TGF-β1	TGF-β1 + Fenofibrate
Intact PGs on Sepharose CL-2B				
K_{av}	0.74 ± 0.02	0.75 ± 0.02	0.72 ± 0.02	0.73 ± 0.02
Cleaved GAG chains on Sepharose CL-6B				
K_{av}	0.470 ± 0.006	0.467 ± 0.003	0.417 ± 0.003	0.430 ± 0.006
*Molecular weight (kDa)	24.2 ± 3.9	24.5 ± 5.9	32.0 ± 0.8	30.0 ± 1.3
Secreted free chains (xyloside-initiated GAGs) on Sepharose CL-6B				
K_{av}	0.63 ± 0.01	0.63 ± 0.02	0.59 ± 0.02	0.60 ± 0.03
*Molecular weight (kDa)	10.7 ± 0.7	10.7 ± 1.1	13.3 ± 1.5	12.5 ± 1.6

Table 3-1

Effect of fenofibrate on vascular SMC proteoglycan and glycosaminoglycan size. The peak K_{av} of intact proteoglycans on Sepharose CL-2B, cleaved GAG chains and secreted free chains on Sepharose CL-6B from SMCs treated with fenofibrate (30 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) as described in Figure 3-20 to 3-22. The data from Wasteson (Wasteson, 1971) was used to convert the K_{av} values to molecular weight for the cleaved chains and secreted free chains. Data are mean±SEM of 3 individual experiments.

3.2.10 Analysis of the fine chemical structure of glycosaminoglycan chains using fluorophore assisted carbohydrate electrophoresis (FACE) analysis – the effect of fenofibrate

It has been recognized that the sulfation pattern of the GAGs may be important for LDL binding (Vijayagopal *et al.*, 1981) and potentially the development and progression of atherosclerosis (Edwards & Wagner, 1988; Schonherr *et al.*, 1993). For example, the GAGs synthesized by aortic smooth muscle cells derived from the atherosclerosis-susceptible White Carneau pigeon show an increase in the 6-sulfation compared to GAGs synthesized by SMCs from the atherosclerosis resistant control (Edwards & Wagner, 1988). Additionally stimulation of monkey aortic smooth muscle cells with the atherogenic growth factor PDGF results in the synthesis of GAGs with a 2-fold increase in the 6:4-sulfate ratio compared to basal cells (Schonherr *et al.*, 1993). Other studies suggest that the degree of sulfation rather than the position of the sulfate groups on the GAGs, determines LDL binding (Sambandam *et al.*, 1991). To investigate a potential structural alteration which may affect lipoprotein binding, we analyzed the sulfation pattern of the disaccharides in the GAGs, synthesized by SMCs in the presence of fenofibrate, using Fluorophore Assisted Carbohydrate Electrophoresis (FACE).

To undertake FACE analysis it is necessary to release the GAGs from the core proteins and digest the GAG chains with chondroitinase ABC, to yield unsaturated disaccharides. Disaccharides are labelled with a fluorophore (2-aminoacridone; AMAC) and resolved by gel electrophoresis. The GAG disaccharides are identified by comparison of their migration with AMAC-labelled disaccharide standards. Gel images are captured using the Glyko imager and analyzed using the Glyko Software.

Fluorophore-tagged disaccharides are separated on high percentage acrylamide gels on the basis of ionic charge, not size (Calabro *et al.*, 2000a; Calabro *et al.*, 2000b). Accordingly, the

disaccharides from hyaluronan which are not sulfated (ΔdiHA) and Δdi0S have a slow electrophoretic migration compared to the Δdi6S (Figure 3-23). The Δdi4S has greater mobility on a FACE gel suggesting that it has a higher charge density than the Δdi6S product (Figure 3-23). Additionally, the uronic acid 2-sulfated disaccharide (ΔdiUA2S) migrates further than the Δdi4S (Figure 3-23).

The majority of disaccharides ($\approx$90%) from CS/DS GAGs synthesized by vascular SMCs are monosulfated either at the 4 position (Δdi4S) or 6 position (Δdi6S) on the galactosamine (Edwards & Wagner, 1988). A small amount ($\approx$10%) of unsulfated disaccharides (Δdi0S), with little or no di-sulfated disaccharides (Δdi4,6S) may be present (Edwards & Wagner, 1988). The CS/DS GAGs synthesized by human vascular SMCs were composed of disaccharides with 7.7$\pm$1.4% Δdi0S (Figure 3-24A), 18.0$\pm$3.7% Δdi6S (Figure 3-24B) and 75.8$\pm$4.6% Δdi4S (Figure 3-24C). A similar sulfation pattern of disaccharides on GAGs synthesized by untreated SMCs was observed for disaccharides isolated from SMCs stimulated with TGF-β1 (2 ng/mL; Figure 3-24A-C). The results with TGF-β1 are similar to the work of Wight and colleagues (Schonherr et al., 1991) who showed that PDGF but not TGF-β1, alters the 4:6-sulfation pattern of GAGs isolated from aortic smooth muscle cells. The extent of the sulfation on th disaccharides did not significantly change when SMCs were treated with fenofibrate (30 μmol/L) either in the presence or absence of TGF-β1 (Figure 3-24A-C). It thus appears that there is little or no effect of fenofibrate treatment on the activity of chondroitin 6- or chondroitin 4-sulfotransferases that lead to the sulfation of GAG chains during proteoglycan biosynthesis by vascular SMCs.
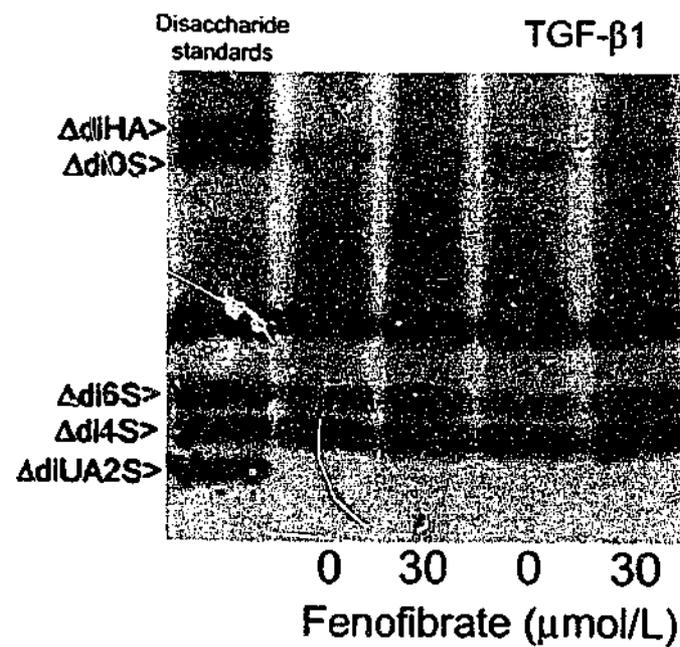
Figure 3-23

Human vascular SMCs treated with fenofibrate show no change in the sulfation pattern of unsaturated disaccharides as assessed by FACE. Human vascular SMCs were treated with fenofibrate (30 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL). The core proteins of the secreted proteoglycans were removed and the GAG chains and samples containing 1-5 μg GAG were digested with chondroitinase ABC. Unsaturated disaccharides were fluorescently labelled with AMAC and separated on a 20% polyacrylamide gel. Fluorescently labelled disaccharide standards (ΔdiHA, Δdi0S, Δdi6S, Δdi4S and Δdi uronic acid (UA)2S) were run in a separate lane. This gel is a representation of results obtained from 3 separate experiments.
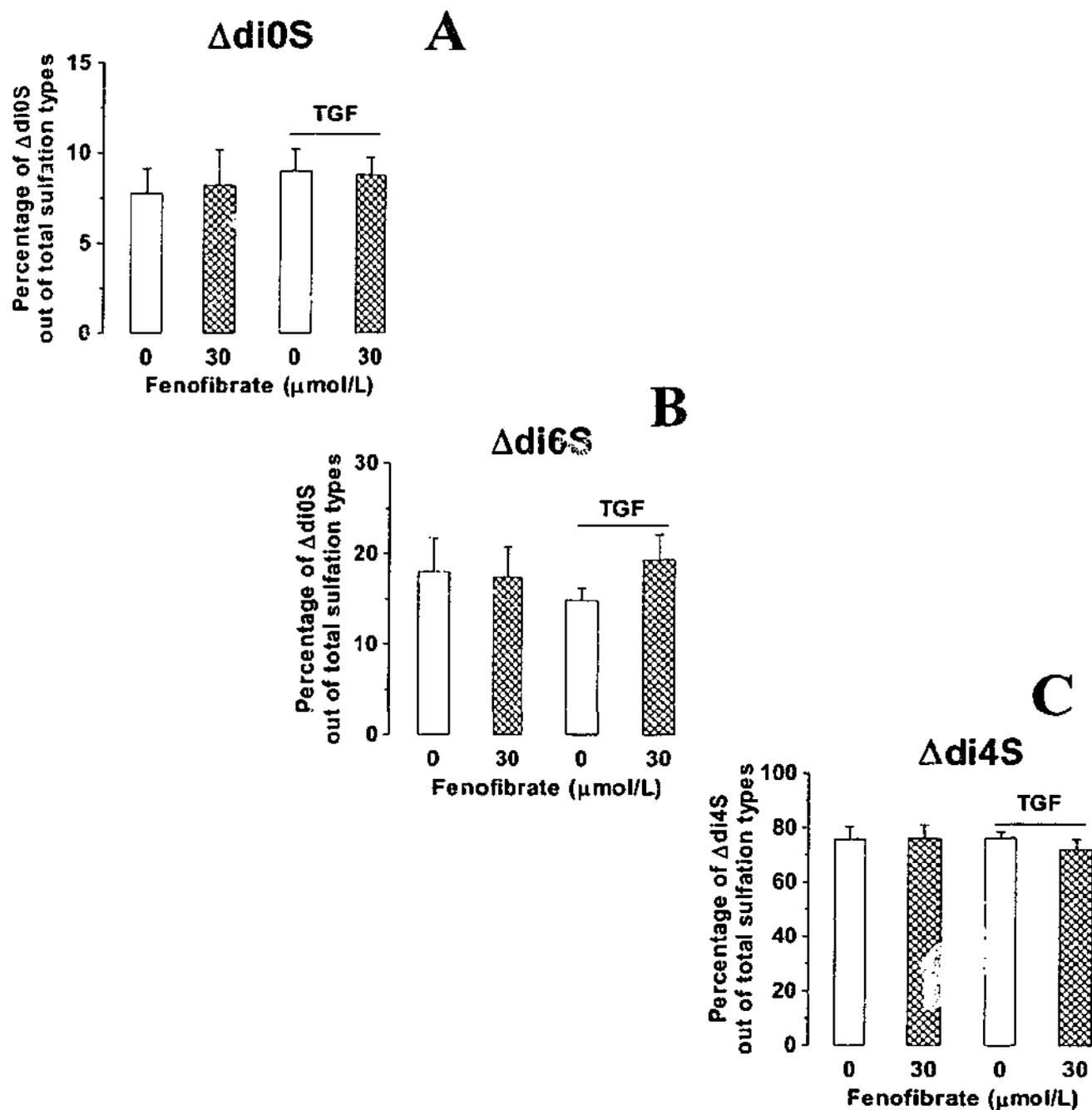
Figure 3-24

Human vascular SMCs treated with fenofibrate show no change in the sulfation pattern of unsaturated disaccharides as assessed by quantitation of FACE gels. Quantitation of (A) ΔdiOS, (B) Δdi6S and (C) Δdi4S from glycosaminoglycans synthesized by vascular SMCs treated with fenofibrate (30 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) and separated by FACE. The density of the disaccharide band indicated, is expressed as a percentage of the combined density of ΔdiOS + Δdi6S + Δdi4S. Each bar represents the mean±SEM from 3 separate experiments performed in duplicate (n=5).

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3.2.11 Differential enzymatic digestion to characterize the GAGs on proteoglycans – the effect of fenofibrate

Multiple properties of the proteoglycans including the type of glycosaminoglycans attached to a proteoglycan can determine the affinity for LDL. For example, recent studies have shown that proteoglycan preparations containing mostly CS and/or DS proteoglycans readily bind to LDL however, heparan sulfate proteoglycans show virtually no binding (Vijayagopal *et al.*, 1996). Additionally, the *in vitro* comparison of LDL binding to CS and DS GAGs, with similar degrees of sulfation, indicates that dermatan sulfate GAG chains, containing mostly iduronic acid, bind to LDL with almost one order of magnitude higher affinity than glucuronic acid containing CS chains to LDL (Cardoso & Mourao, 1994; Gigli *et al.*, 1993). Pharmacological regulation of vascular SMCs to synthesize proteoglycans with less DS and consequently more CS containing GAGs would be a potential therapeutic mechanism to reduce the retention of LDL in the vasculature.

Human vascular SMCs treated with fenofibrate (30 µmol/L) and/or those maintained under basal conditions were metabolically labelled with $[^{35}S]$-sulfate. Secreted proteoglycans were isolated and equal amounts $(20 \times 10^3$ cpm) were separately digested with chondroitinase AC, chondroitinase B, chondroitinase ABC and heparitinase. The samples were separated by SDS-PAGE (Figure 3-25) and the digested proteoglycans were classified as chondroitin sulfate, dermatan sulfate, both CS/DS and heparan sulfate, respectively. For a full analysis of GAG composition, keratanase was included in the protocol of GAG digestion. The enzyme-resistant, radiolabelled proteoglycans were profiled using Image Quant 5.1 (Figure 3-26). A proteoglycan sample that was not treated with any enzymes was included on the gel and used to compare to the digested samples. The density of non-enzyme treated bands was used to calculate the percentage digestion of the corresponding band in the enzyme treated

lanes (Table 3-2).

Band 1 is the largest of the proteoglycans and remains in the stacking gel (Figure 3-25). Under basal conditions, 88.0% of band 1 was digested with C'ase AC, which was complemented by 66.0% digestion with C'ase ABC (Table 3-2). There was no digestion of band 1 with C'ase B (Table 3-2). Within band 1, 56.8% was digested with heparitinase and 25.2% with keratanase (Table 3-2). This information indicates that band 1 contains a mixture of a CS proteoglycan and an HS proteoglycan. It is known that versican is a CSPG while perlecan exists with two HS GAGs and one CS GAG (Costell *et al.*, 1997; Dolan *et al.*, 1997; Groffen *et al.*, 1996; Kokenyesi & Silbert, 1995). Band 1 may contain both versican and perlecan however, it is unknown whether or not human vascular SMCs express a large KSPG. Proteoglycan(s) within band 1 from vascular SMCs treated with fenofibrate showed 82.2% digestion with C'ase AC, 72.1% digestion with C'ase ABC and minimal digestion (7.5%) with C'ase B, indicating that this band is mostly a CSPG (Table 3-2). There was no digestion of band 1 with heparitinase or keratanase from SMCs treated with fenofibrate (Table 3-2) suggesting that fenofibrate may have reduced the synthesis of the HSPG component of band 1 that was produced by untreated cells.
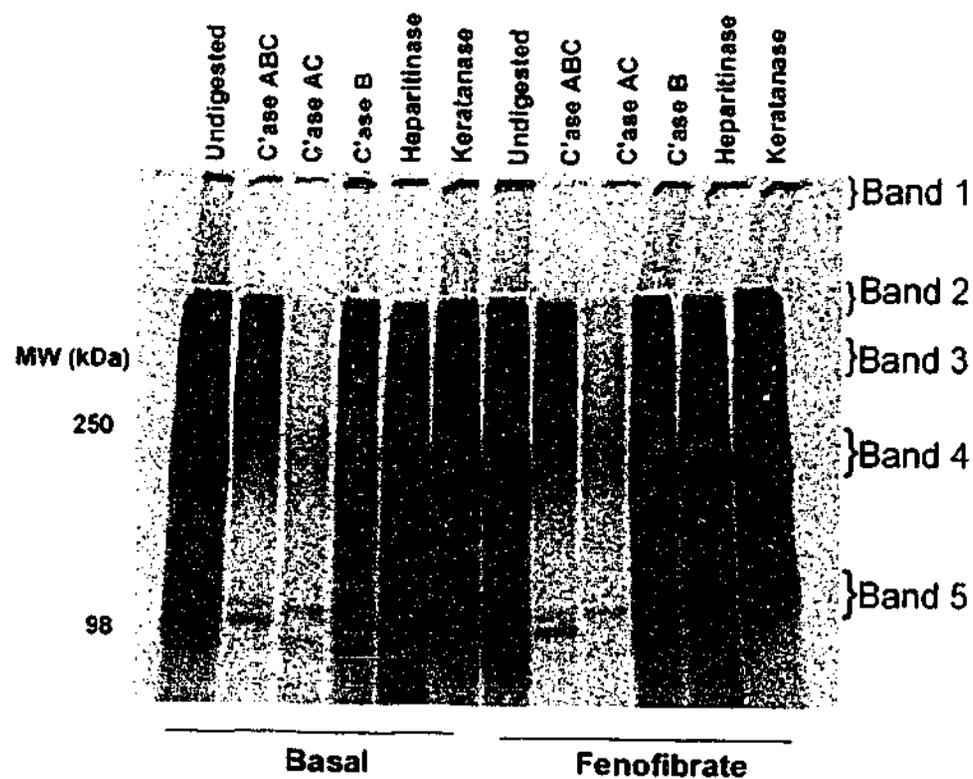
Figure 3-25

Human vascular SMCs synthesize predominantly CS/DS proteoglycans. Vascular SMCs were treated with and without fenofibrate (30 μmol/L) and metabolically labelled with [^{35}S]-sulfate. Proteoglycans were isolated and subjected to digestion with chondroitinase (C'ase) ABC, C'ase AC, C'ase B, heparitinase, and keratanase. Digested samples and an undigested control for each treatment were separated on 4-13% linear gradient SDS-PAGE. Non-radiolabelled molecular weight protein markers were run in separate lane. These data represent 4 gels with similar results.
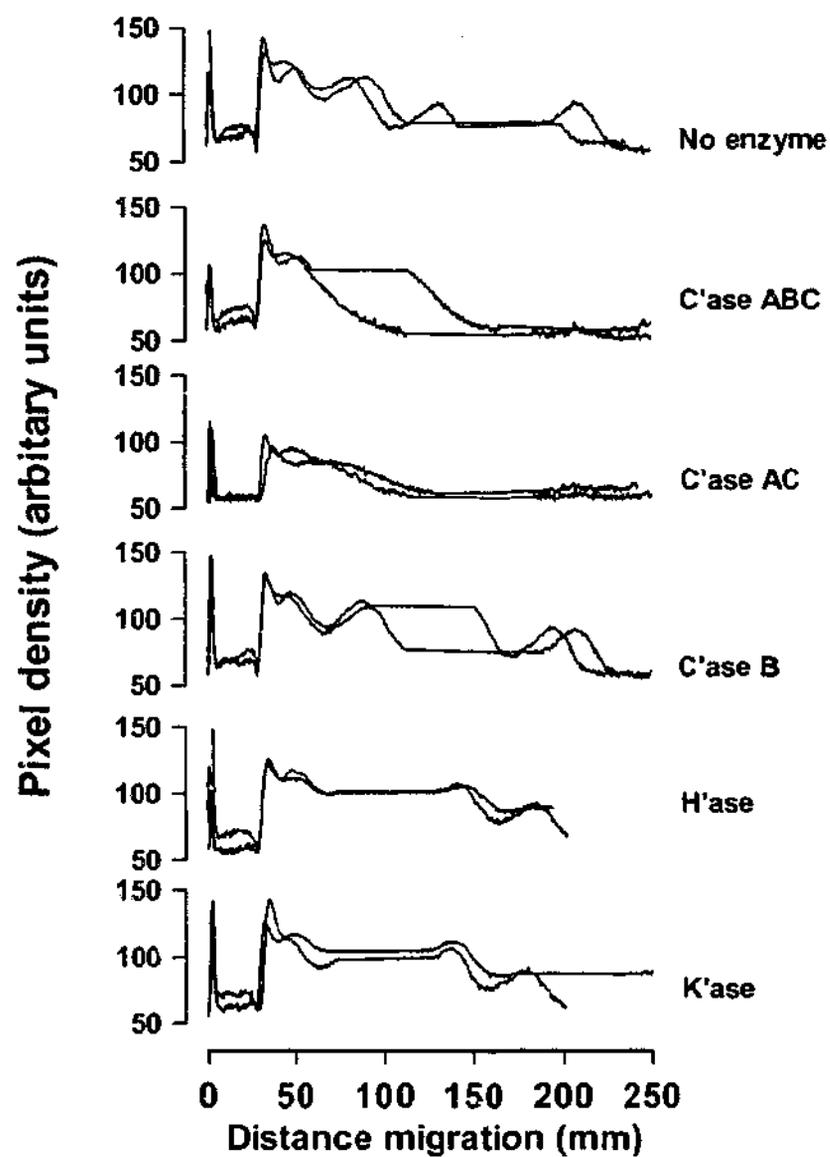
Figure 3-26

Densitometric profiles of proteoglycans from control and fenofibrate treated human vascular SMCs following treatment with GAG digestion enzymes and separation by SDS-PAGE. Proteoglycans secreted from untreated vascular SMCs (black line) or SMCs treated with fenofibrate (30 μmol/L; blue line) were subjected to digestion with chondroitinase (C'ase) ABC, C'ase AC, C'ase B, heparitinase (H'ase), and keratanase (K'ase) and separated by SDS-PAGE. Each lane (including the stacking gel) from the SDS-PAGE gel from Figure 3-25 was scanned for pixel density. These data represent 4 gels with similar results.

Band 2 is a large proteoglycan which enters the resolving gel but does not migrate (Figure 3-25). Vascular SMCs maintained under basal conditions showed that proteoglycans within band 2 are susceptible to C'ase ABC digestion showing 18.6% digestion with this enzyme (Table 3-2). Chondroitinase AC digested 51.6% of band 2 however, no digestion of band 2 was observed following digestion with C'ase B (Table 3-2). These data indicate that the proteoglycan(s) within band 2 contain a small amount of CS GAGs but do not contain DS GAGs. Heparitinase showed the most activity towards band 2 with 62.2% digestion, while only a small amount (18.8%) of band 2 was susceptible to digestion with keratanase (Table 3-2). These data suggest that band 2 may also contain the HS proteoglycan, perlecan with a lower molecular weight compared to that observed in band 1. The reduction in perlecan molecular weight might be due to a variable number/length of HS/CS GAG chains (Iozzo, 1998). Unlike proteoglycans from untreated SMCs, proteoglycans secreted from vascular SMCs treated with fenofibrate showed that band 2 was mostly susceptible to C'ase AC (73.6% digested) and 30.4% was digested with C'ase ABC (Table 3-2). As observed with proteoglycans in band 2 from cells maintained under basal conditions, proteoglycans in band 2 from cells treated with fenofibrate showed minimal digestion (9.1%) with C'ase B indicating that this band contains proteoglycan(s) with mostly CS GAGs (Table 3-2). Proteoglycans within band 2 from SMCs treated with fenofibrate showed 41.3% digestion with heparitinase and minimal digestion (10.6%) with keratanase (Table 3-2). These data show that the proteoglycans from human vascular SMCs synthesized under basal conditions which appear as band 2 on SDS-PAGE are a mixture of HSPGs and CSPGs whereas the proteoglycans in band 2 from cells treated with fenofibrate contain mostly CS GAGs. This data suggest that fenofibrate treatment of vascular SMCs reduces the HSPG component of band 2.

		% Proteoglycans digested				
		C'ase				
		ABC	AC	B	H'ase	K'ase
Band 1	Control	63.0	88.0	26.0	56.8	25.2
(versican;	Fenofibrate	72.1	82.2	7.5	0	0
CSPG)						
Band 2	Control	18.6	51.6	0	62.2	18.8
(perlecan;	Fenofibrate	30.4	73.6	9.1	41.3	10.6
HS/CSPG)						
Band 3	Control	39.3	67.7	8.2	41.1	0
(HS/CSPG)	Fenofibrate	38.9	74.3	20.5	31.4	31.3
Band 4	Control	81.7	80.7	23.6	43.3	7.5
(biglycan;	Fenofibrate	85.8	88.0	33.1	30.7	31.5
CS/DSPG)						
Band 5	Control	84.4	80.0	24.4	0	6.5
(decorin;	Fenofibrate	85.1	86.3	15.3	0.9	11.2
CS/DSPG)						

Table 3-2

Composition of [^{35}S]-sulfate-labelled proteoglycans separated by SDS-PAGE using enzyme digestion. Results are the percentage of proteoglycan within a particular band (band 1-5, see Figure 3-25) digested with Chondroitinase ABC (C'ase ABC), C'ase AC, C'ase B, heparitinase (H'ase) or Keratanase (K'ase). Each value is the mean of 2-4 different gels.

Human vascular SMCs synthesize a large proteoglycan (>250 kDa) which enters the separating gel, labelled band 3 (Figure 3-25). Under basal conditions band 3 showed 67.7% digestion with C'ase AC and 39.3% was digested with C'ase ABC, however there was little (8.2%) digestion of band 3 with C'ase B (Table 3-2). These data indicate that band 3 contains proteoglycans with mostly CS GAGs but may not contain DS GAGs. An appreciable amount (41.1%) of band 3 was digested with heparitinase but no digestion of band 3 was observed with keratanase (Table 3-2). These data indicate that band 3 contains CSPGs and HSPGs and these may due to a proteoglycan dimer (Iozzo, 1998). Proteoglycans within band 3 from vascular SMCs treated with fenofibrate were mostly susceptible to digestion with 74.3% digestion following C'ase AC treatment, 38.9% digestion following C'ase ABC treatment and 20.5% digestion with C'ase B treatment (Table 3-2). Proteoglycans from fenofibrate treated SMCs showed appreciable amounts of HSPG (31.4%) and KSPG (31.3%; Table 3-2). Similar to the analysis of band 2, these data show that the treatment of vascular SMCs with fenofibrate increases the amount of CS proteoglycans and decreases HS proteoglycans within band 3. Additionally DS and KS containing proteoglycans appear within band 3 following treatment of SMCs with fenofibrate which was not observed in band 3 from untreated cells.

The proteoglycans contained within band 4 have a molecular weight range of 180-230 kDa on SDS-PAGE (Figure 3-25). Vascular SMCs maintained under basal conditions showed that band 4 was mostly susceptible to digestion with C'ase AC (80.7%) and C'ase ABC (81.7%) while some of band 4 (24.6%) was digested with C'ase B (Table 3-2). An appreciable amount (43.3%) of band 4 was digested with heparitinase with little (7.5%) digestion with keratanase (Table 3-2). The molecular weight and GAG digestion data indicate that band 4 contains mostly biglycan with CS/DS GAG chains. It is unknown why a proportion of band 4 is susceptible to digestion with heparitinase (Table 3-2). Vascular SMCs treated with fenofibrate

show that proteoglycans within band 4 were mostly susceptible to digestion with C'ase AC (88.8%) and C'ase ABC (90.5%), while a small amount (9.7%) of this band was digested with C'ase B (Table 3-2). Under these conditions 26.6% of band 4 was digestible with heparitinase and 28.6% with keratanase (Table 3-2). These data indicate that the treatment of vascular SMCs with fenofibrate compared to untreated cells, slightly increases the CSPG component of band 4 while reducing the DS and HS components in addition to the appearance of proteoglycan containing KS GAGs.

The proteoglycan band with a molecular weight range of 80-120 kDa is labelled band 5 (Figure 3-25). Vascular SMCs maintained under basal conditions showed that band 5 was mostly susceptible to digestion with C'ase AC (80.0%) and C'ase ABC (84.4%) and a small amount (24.4%) was digested with C'ase B (Table 3-2). Band 5 was not digestible with heparitinase or keratanase (Table 3-2). The molecular weight and the GAG composition data indicate that band 5 contains mostly decorin with CS/DS chains. Vascular SMCs treated with fenofibrate showed 86.3% digestion of band 5 with C'ase AC treatment, 85.1% digestion with C'ase ABC tr· .ent and 15.3% digestion with C'ase B treatment (Table 3-2). As observed with proteoglycans from untreated cells, the proteoglycan within band 5 from cells treated with fenofibrate was not susceptible to digestion with heparitinase or keratanase (Table 3-2). These data indicate that treatment of vascular SMCs with fenofibrate may induce a small reduction in the DS component of decorin, compared to cells maintained under basal conditions.

Vascular SMCs express osteoglycin, a KS proteoglycan (Fernandez *et al.*, 2003; Shanahan *et al.*, 1997). The level of digestion of proteoglycans within bands 1-5 with keratanase was somewhat surprising and the effect of fenofibrate to increase the KS component was unexpected but consistent. These data may arise from an effect of fenofibrate treatment to

increase KS GAGs or from non-specific enzyme activity. It is possible that the keratanase preparation may contain other GAG digesting enzymes such as chondroitinase. Nevertheless, the marked differences between keratanase digested proteoglycans from fenofibrate and non-treated vascular SMCs are apparent, which may be interesting but were not followed up at this time.

In summary, the characterization of GAGs by enzyme digestion showed that the versican and decorin bands (on SDS-PAGE) from vascular SMCs treated with fenofibrate contained less dermatan sulfate (DS) GAGs than the decorin and versican bands isolated from untreated cells. Dermatan sulfate GAGs arise from the epimerization of the chondroitin (CS) chain by the glucuronyl C5-epimerase in the *trans*-Golgi network (Malmstrom & Fransson, 1975). The effect of fenofibrate to reduce DS containing GAGs on versican/decorin may be due to an inhibition of the epimerase as this is the limiting factor in DS biosynthesis (Tiedemann *et al.*, 2001).

3.2.12 Time course of effects on sulfate incorporation into GAGs – the effect of fenofibrate

Fibrates/PPAR-α ligands have been suggested to have two potential actions; acute non-genomic actions and genomic actions dependent upon regulation of transcription. Although a detailed analysis of the temporal aspects of the action of fenofibrate on proteoglycan synthesis is outside the scope of this thesis, an initial approach is presented here. These experiments are somewhat compromised by the practical fact that a radiolabelled proteoglycan takes $\approx$2-3 h to be synthesized and secreted (Fellini *et al.*, 1984), which is an appreciable amount of time when considering the genomic versus non-genomic effects of fibrates/PPAR-α ligands. Nevertheless, the effect of fenofibrate treatment of vascular SMCs in

the presence and absence of TGF-β1 was tested at different time intervals. Additionally, we investigated whether or not the effect of fenofibrate treatment to reduce proteoglycan synthesis and GAG length was an irreversible response or whether this effect could be reversed following removal of fenofibrate treatment.

For the time related experiments, human vascular SMCs were seeded on 24-well plates at 40×10^3 cells per well and grown to confluence for 3 days. Cells were serum deprived for 48 h then treated with fenofibrate (30 μmol/L) for 0, 3, 6, 12 and 24 hours. TGF-β1 (2 ng/mL) and [^{35}S]-sulfate were added for a further 12 hours following each treatment time. For the recovery response experiments, human vascular SMCs were pre-treated with fenofibrate (30-50 μmol/L) for 6 h prior to the addition of TGF-β1 (2 ng/mL) for 24 h. The treatment medium was removed and replaced with control medium (0.1% DMSO) for 24 h, prior to metabolic labelling with [^{35}S]-sulfate for a further 24 h. Secreted proteoglycans were quantitated by the CPC precipitation assay and the electrophoretic migration was assessed by SDS-PAGE.

Vascular SMCs exposed to fenofibrate (30 μmol/L) for 24 h prior to metabolic labelling, synthesized 30.4% ($P<0.001$) less proteoglycans compared to SMCs that were treated with fenofibrate and metabolically labelled at the same time (0 h time point; Figure 3-27). The pre-treatment of vascular SMCs with fenofibrate for 12 h and 24 h followed by the addition of TGF-β1, significantly inhibited [^{35}S]-sulfate incorporation into proteoglycans by 16.2% ($P<0.05$) and 17.1% ($P<0.05$), respectively compared to cells that were treated with TGF-β1 alone with no prior exposure to fenofibrate (Figure 3-28). The temporal aspects of the effect of fenofibrate followed by treatment with TGF-β1, on GAG elongation were further evaluated by SDS-PAGE. The reduction in [^{35}S]-sulfate incorporation into proteoglycans over time following treatment of vascular SMCs with fenofibrate, correlated with an increased

electrophoretic mobility of the proteoglycans by SDS-PAGE (Figure 3-29A and B). The apparent reduction in GAG length was observed following treatment of vascular SMCs with fenofibrate for 6 h prior to metabolic labelling, in the presence TGF-β1 (Figure 3-29A and B). The results indicate that the effect of fenofibrate to reduce [^{35}S]-sulfate incorporation into GAG chains and to inhibit GAG elongation, is time dependent.
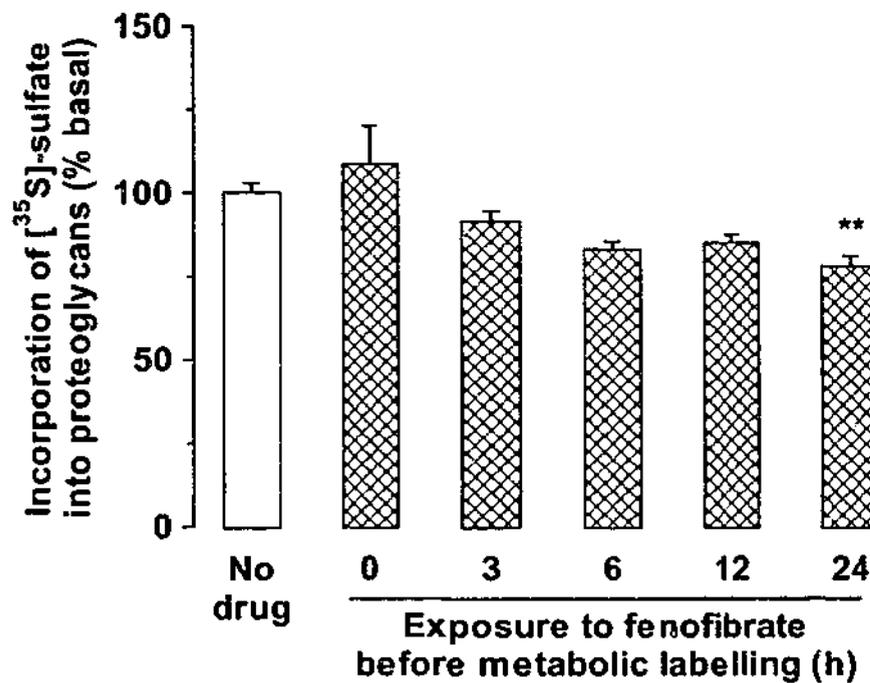
Figure 3-27

Fenofibrate treatment of human vascular SMCs causes a time-dependent decrease in [^{35}S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with fenofibrate (30 μmol/L) for 3-24 h prior to metabolic labelling with [^{35}S]-sulfate for a further 12 h. Proteoglycans were quantitated using the CPC precipitation assay and results are expressed as mean±SEM of 2 experiments performed in quadruplicate wells (n=8, ** $P<0.01$ versus no drug). Pre-normalized basal value is 126.7±8.2 cpm [^{35}S]-sulfate/10^3 cells.
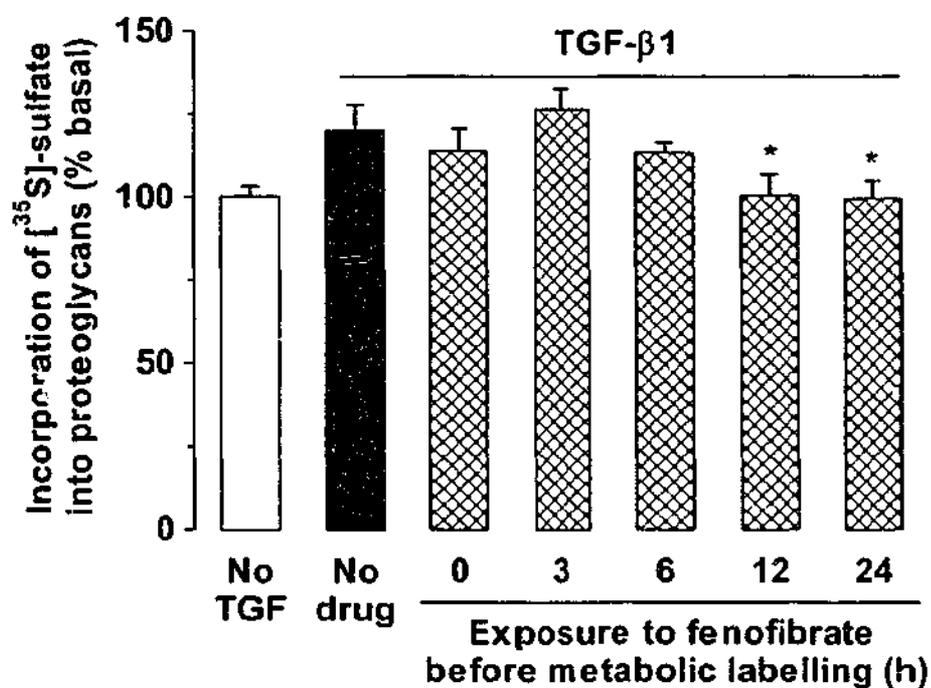
Figure 3-28

Fenofibrate treatment of human vascular SMCs in the presence of TGF-β1 causes a time-dependent decrease in [^{35}S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with fenofibrate (30 μmol/L) for 3-24 h followed by the addition of TGF-β1 (2 ng/mL) and metabolic labelling with [^{35}S]-sulfate for a further 12 h. Proteoglycans were quantitated using the CPC precipitation assay and the results are expressed as mean±SEM of 2 experiments performed in quadruplicate wells (n=8, * $P<0.05$ versus TGF-β1 no drug). Pre-normalized basal value is 126.7±8.2 cpm [^{35}S]-sulfate/10^3 cells.

A

Treatment with
30 μmol/L fenofibrate (h)

No drug 0 3 6 12 24

Treatment with
30 μmol/L fenofibrate (h)

No drug 0 3 6 12 24

MW (kDa)

250

98

Versican

CSPG

Biglycan

Decorin

TGF-β1

B

Pixel density

Con

FFB (6h)

FFB (24h)

TGF-β1

FFB (6h) + TGF-β1

FFB (24h) + TGF-β1

0   5   10   15

Distance migration (mm)
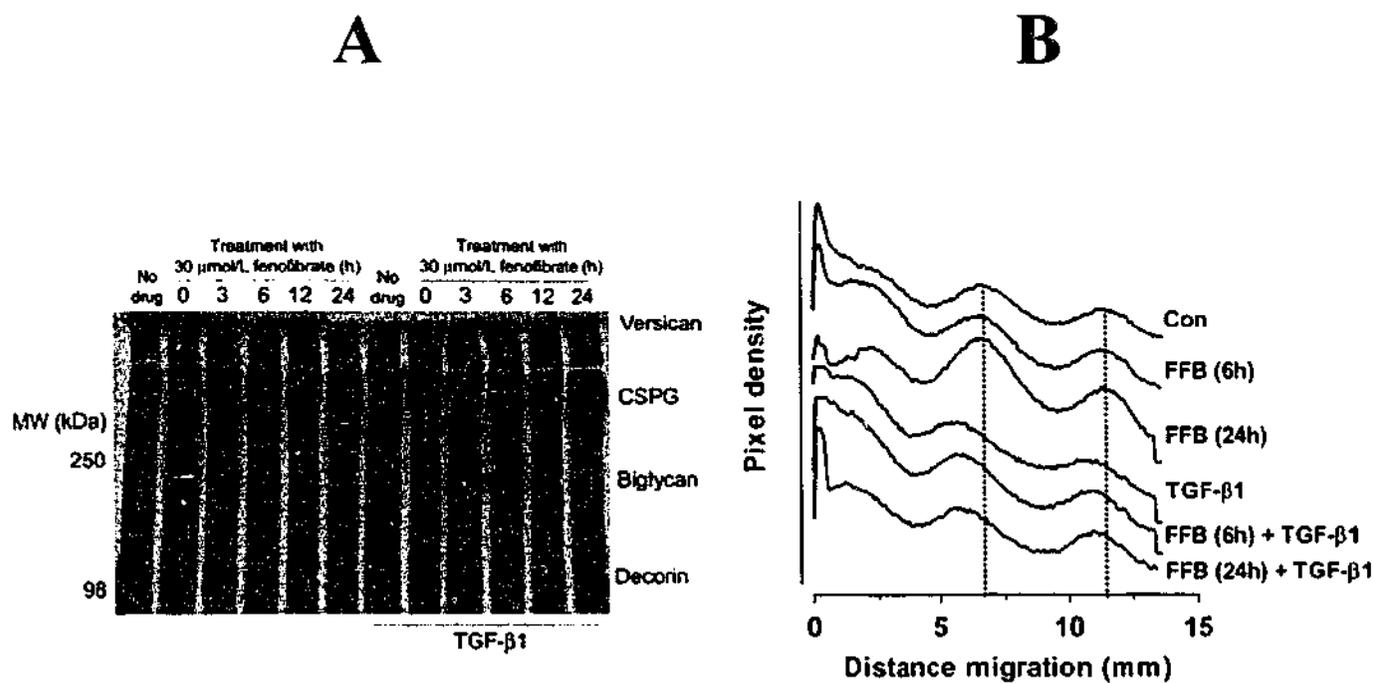
Figure 3-29

Time dependent increase in the electrophoretic mobility of proteoglycans secreted from fenofibrate treated human vascular SMCs. Human vascular SMCs were treated with fenofibrate (30 μmol/L) for 3-24 h prior to the addition of TGF-β1 (2 ng/mL) to some cell and metabolic labelling of all cells with [^{35}S]-sulfate for a further 12 h. (A) Secreted proteoglycans were separated by SDS-PAGE. (B) Graphical representation of the migration of radiolabelled proteoglycans from control (Con, 0.1% DMSO), fenofibrate (FFB, 30 μmol/L), TGF-β1 and fenofibrate plus TGF-β1 treated SMCs for 6 h or 24 h from the gel presented in A. Dotted lines are the peaks of biglycan and decorin from untreated SMCs. Gel is representative of 2 independent experiments. To facilitate data analysis, scans were strategically positioned to reflect the order of appearance on the gel using Fig.P Version 2.98.

We also addressed the question as to whether or not the reduction in vascular proteoglycan synthesis and GAG length observed with fenofibrate treatment, was reversible. It was observed that the treatment of vascular SMCs with fenofibrate (50 μmol/L) followed by removal of fenofibrate showed a 2-fold increase ($P<0.05$) in [^{35}S]-sulfate incorporation into proteoglycans compared to SMCs that did not have fenofibrate removed (Figure 3-30). Vascular SMCs treated with fenofibrate in the presence of TGF-β1 that were allowed to recover from fenofibrate treatment, only partially returned to the proteoglycan synthesis levels observed with TGF-β1 stimulation alone, 146.9 ± 17.9 cpm/10^3 cells compared to 443.3 ± 87.7 cpm/10^3 cells, respectively (Figure 3-30). The electrophoretic mobility of proteoglycans synthesized by vascular SMCs that had the fenofibrate treatment removed was similar to proteoglycans from untreated SMCs (Figure 3-31). The electrophoretic mobility of proteoglycans from cells that were allowed to recover from fenofibrate treatment in the presence of TGF-β1, had migrated further that the proteoglycans synthesized in the presence of TGF-β1 alone (Figure 3-31). These data indicate that the effect of fenofibrate treatment on vascular SMCs is at least partially reversible and this is consistent with a mechanistic action on GAG elongation processes in vascular smooth muscle.
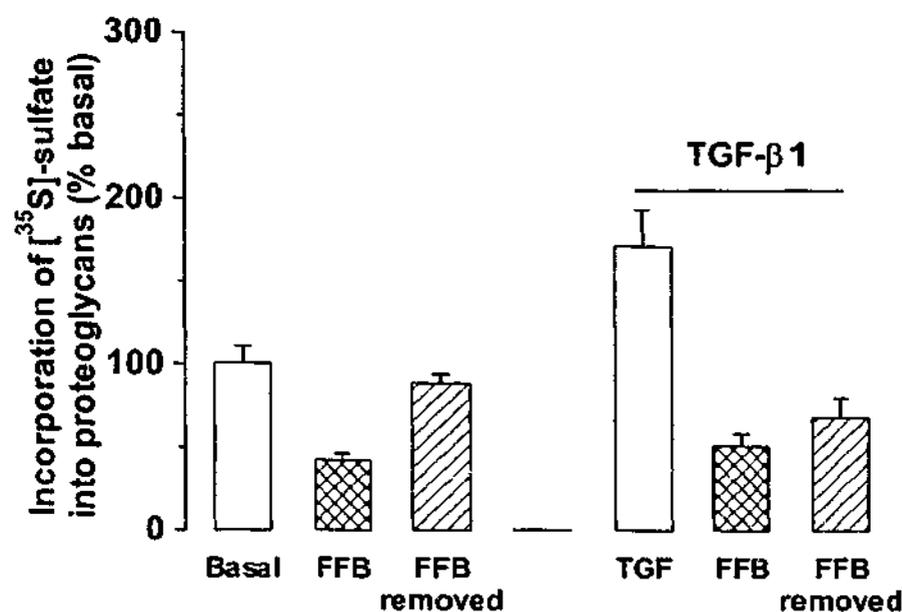
Figure 3-30

The reduction in [^{35}S]-sulfate incorporation into proteoglycans following fenofibrate treatment of SMCs is reversible. Human vascular SMCs were treated with fenofibrate (50 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) for 24 h and were washed free of fenofibrate. Vascular SMCs were metabolically labelled with [^{35}S]-sulfate and assessed for sulfate incorporation into proteoglycans by the CPC precipitation assay. Data are expressed as the mean±SEM of 2 experiments performed in triplicate/quadruplicate (n=5-8). Pre-normalized basal value is 243.5±89.0 cpm [^{35}S]-sulfate/10^3 cells.

A

TGF-β1

Basal | FFB | FFB removed | TGF | FFB | FFB removed

Versican
CSPG

MW (kDa)

250 — Biglycan

98 — Decorin

B

Pixel density

Con
FFB
FFB removed
TGF-β1
FFB+TGF-β1
FFB + TGF removed

0   5   10   15   20
Distance migration (mm)
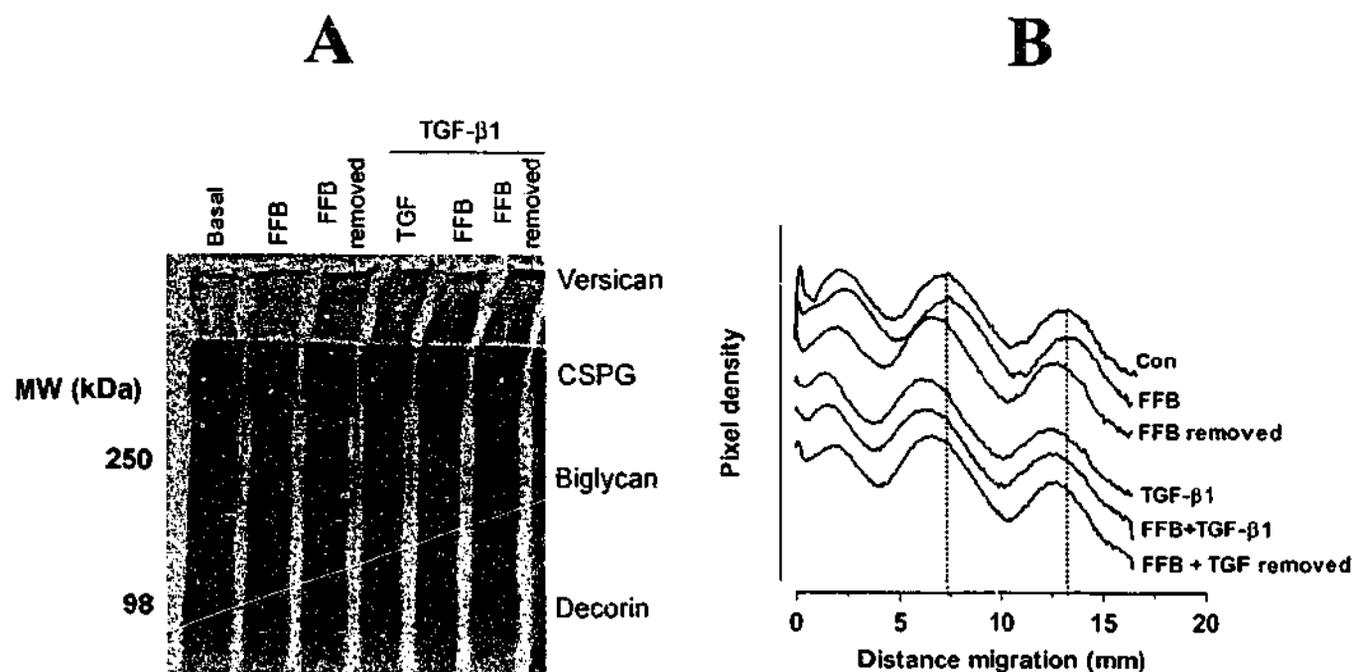
Figure 3-31

The increase in electrophoretic mobility of proteoglycans secreted from fenofibrate treated vascular SMCs is reversible. Human vascular SMCs were treated with fenofibrate (50 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) for 24 h and then washed free of fenofibrate. (A) Vascular SMCs were metabolically labelled with [^{35}S]-sulfate and secreted proteoglycans were separated by SDS-PAGE. (B) Graphical representation of the migration of radiolabelled proteoglycans from control (Con, 0.1% DMSO), fenofibrate (FFB, 50 μmol/L), TGF-β1 or fenofibrate plus TGF-β1 treated SMCs with or without removal of fenofibrate, from the gel presented in A. Dotted lines are peak migration of biglycan and decorin from untreated SMCs. Gel is representative of 2 independent experiments. To facilitate data analysis, scans were strategically positioned to reflect order of appearance on the gel using Fig.P Version 2.98.

3.2.13 Chemical modification of various PPAR-α ligands - the effect on proteoglycan synthesis

It is known that fenofibric acid is the actual PPAR-α ligand while fenofibrate (an ester) is the pro-drug (Balfour *et al.*, 1990), but the latter would be more permeable to SMCs than the active ligand. The relative activity or potency of the two agents on non-genomic effects is unknown. An amide of fenofibrate may be equally permeable to the cell as fenofibrate but its hydrolysis as an amide is much slower than the ester hence, fenofibrate-amide may be less active towards PPAR-α than fenofibrate or fenofibric acid. We hypothesized that the effect of fenofibrate to reduce glycosaminoglycan length may be related to cell permeability. We assumed that the level of permeability of PPAR ligands to the nucleus would be in the order of ester≈amide>acid and PPAR-α activity would be acid>ester>amide. Gemfibrozil has similar actions to fenofibrate on vascular SMC proteoglycan synthesis and GAG length (Nigro *et al.*, 2002). To extend our investigation of cell permeability versus PPAR-α ligand activity, gemfibrozil (an acid) was included in the study by developing gemfibrozil-ester and gemfibrozil-amide. Fenofibrate (F-ester), fenofibric acid (F-acid) and gemfibrozil (G-acid) were available to us however, fenofibrate amide (F-amide), gemfibrozil ester (G-ester) and gemfibrozil amide (G-amide) were designed by Dr Peter Little (Baker Heart Research Institute, Melbourne) and chemically synthesized by Dr Ian Crosby and colleagues (Victorian College of Pharmacy, Monash University) using the N-*1*-benzotriazolecarboxylic acid chloride reaction (Lovrek *et al.*, 2000), to test our hypothesis.

The effects of F-amide, G-ester and G-amide on proteoglycan synthesis by human vascular SMCs were studied at 20 µmol/L because in separate experiments it was observed that concentrations above 30 µmol/L lead to crystallization of the synthesized compounds and increased cell loss, as assessed by phase contrast microscopy and cell counting, respectively.

The effects of chemically modified fenofibrate and gemfibrozil were studied in the presence and absence of TGF-β1 (1 ng/mL) and PDGF (50 ng/mL).

Vascular SMCs treated with F-ester or F-acid did not show any significant change in [^{35}S]-sulfate incorporation into proteoglycans (Figure 3-32). Treatment of vascular SMCs with F-amide (20 μmol/L) decreased [^{35}S]-sulfate incorporation into proteoglycans by 27.0% ($P<0.01$, Figure 3-32). Stimulation of vascular SMCs with PDGF (50 ng/mL), increased [^{35}S]-sulfate incorporation into proteoglycans from 120.2±13.6 cpm/10^3 cells from untreated cells to 231.5±31.8 cpm/10^3 cells ($P<0.001$) in the presence of PDGF (Figure 3-32). In the presence of PDGF and F-ester (20 μmol/L) there was 8.5% inhibition (not significant) of [^{35}S]-sulfate incorporation into proteoglycans however F-acid (20 μmol/L) had no effect on [^{35}S]-sulfate incorporation into proteoglycans compared to cells treated with PDGF alone (Figure 3-32). Incorporation of [^{35}S]-sulfate into vascular proteoglycans was inhibited by 29.6% ($P<0.001$) in the presence of F-amide with PDGF compared to cells treated with PDGF alone (Figure 3-32). Vascular SMCs stimulated with TGF-β1 showed a 36.4% ($P<0.001$) increase in [^{35}S]-sulfate incorporation into proteoglycans compared to untreated cells (Figure 3-32). In the presence of TGF-β1, F-ester (20 μmol/L) tended to decrease (by 10.1%, not significant) [^{35}S]-sulfate incorporation into proteoglycans, while F-acid had a minimal effect on [^{35}S]-sulfate incorporation into proteoglycans compared to cells treated with TGF-β1 alone (Figure 3-32). As observed under basal conditions and in the presence of PDGF, F-amide treatment in the presence of TGF-β1 decreased [^{35}S]-sulfate incorporation into proteoglycans to 118.9±13.4 cpm/10^3 cells compared to 157.3±10.4 cpm/10^3 cells from cells treated with TGF-β1 alone ($P<0.001$, Figure 3-32).

Analysis of the electrophoretic mobility of proteoglycans showed that F-ester, F-acid and F-amide treated vascular SMCs synthesized proteoglycans with a similar migration

pattern by SDS-PAGE however, these proteoglycans had migrated further than the proteoglycans from untreated SMCs (Figure 3-33A and B). Analysis of the electrophoretic mobility of proteoglycans synthesized in the presence of PDGF showed a slower migration by SDS-PAGE than proteoglycans from untreated cells indicating an increase in GAG length (Figure 3-33A). Proteoglycans from vascular SMCs treated with F-ester and F-acid in the presence of PDGF, had an increased electrophoretic migration by SDS-PAGE compared to cells treated with PDGF alone (Figure 3-33A and C). The reduction in sulfate incorporation into proteoglycans following treatment of vascular SMCs with F-amide treatment in the presence of PDGF, was not associated with an increase in the electrophoretic migration by SDS-PAGE; these proteoglycans had a similar mobility to proteoglycans from cells treated with PDGF alone (Figure 3-33A and C). The electrophoretic mobility of proteoglycans from SMCs stimulated with TGF-β1 (1 ng/mL) did not show a marked reduction in electrophoretic mobility compared to proteoglycans from untreated cells (Figure 3-33A). In the presence of TGF-β1 and F-ester, the migration pattern of vascular proteoglycans was not altered compared to proteoglycans from cells treated with TGF-β1 alone (Figure 3-32A and D). Proteoglycans synthesized in the presence of TGF-β1 and F-acid or F-amide showed a reduced mobility by SDS-PAGE compared to cells treated with TGF-β1 alone (Figure 3-33A and D).
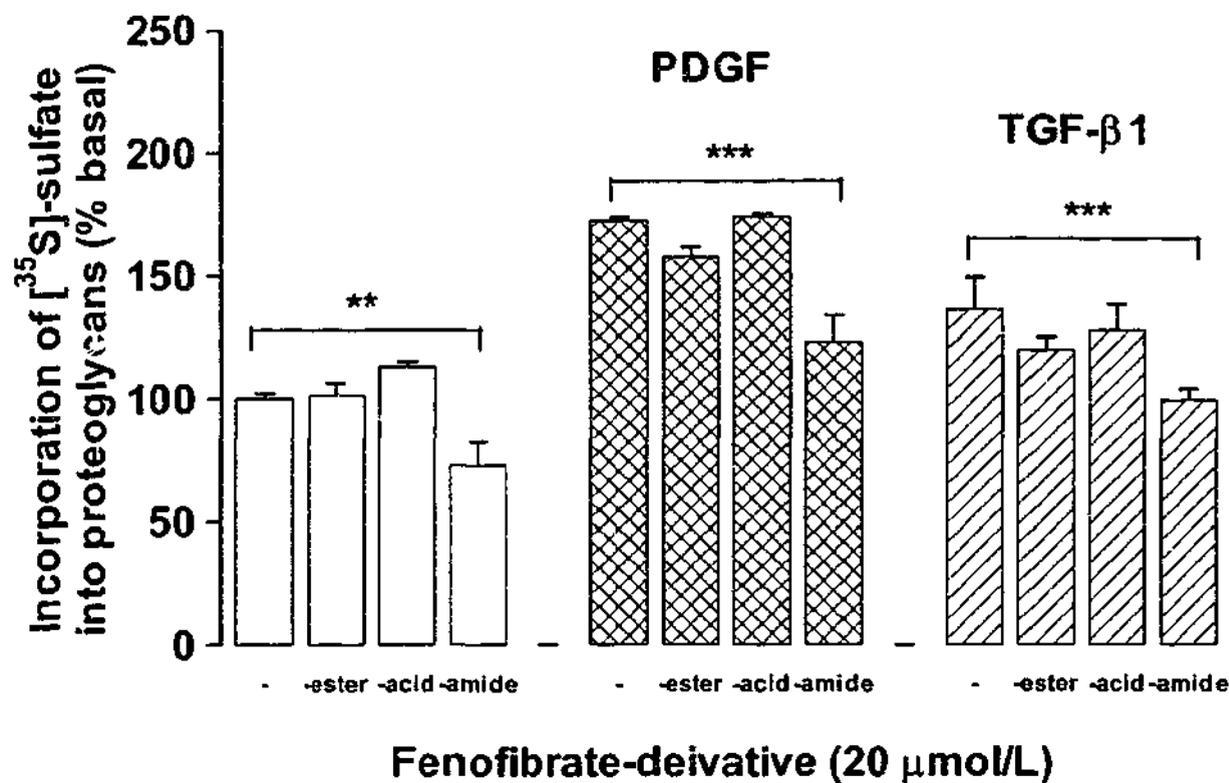
Figure 3-32

Fenofibrate-amide reduces [^{35}S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with 0.1% DMSO (-) or fenofibrate-ester, -acid or -amide (each at 20 μmol/L) in the presence and absence of TGF-β1 (1 ng/mL) or PDGF (50 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Incorporation of [^{35}S]-sulfate into proteoglycans was assessed by the CPC precipitation assay. Results are mean±SEM of 2 experiments performed in triplicate (** $P<0.01$, *** $P<0.001$). Pre-normalized basal value is 120.2±13.6 cpm [^{35}S]-sulfate/10^3 cells.

A Fenofibrate-derivative

- -es -ac -am - -es -ac -am - -es -ac -am

MW (kDa)

250

98

Versican

HS/CSPG

Biglycan

Decorin

PDGF   TGF-β1

B Fenofibrate-derivative treated SMCs

C PDGF + fenofibrate-derivative treated SMCs

D TGF-β1 + fenofibrate-derivative treated SMCs
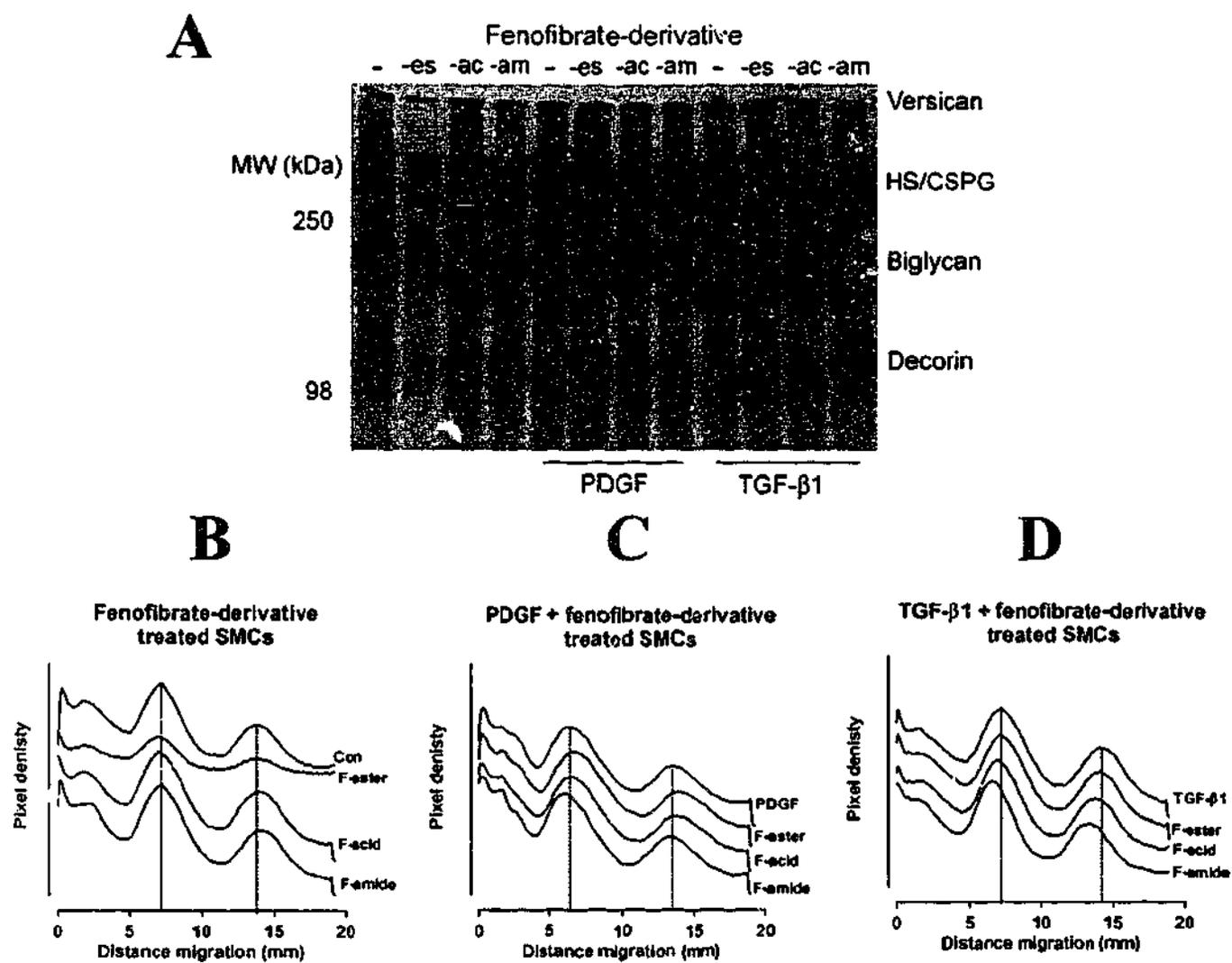
Figure 3-33

Fenofibrate-ester treatment of human vascular SMCs increases the electrophoretic mobility of secreted proteoglycans. Human vascular SMCs were treated with 0.1% DMSO (- or Con) or fenofibrate-ester (-es; F-ester), -acid (-ac; F-acid) or -amide (-am; F-amide), each at 20 μmol/L, in the presence and absence of TGF-β1 (1 ng/mL) or PDGF (50 ng/mL) and metabolically labelled with [^{35}S]-sulfate. (A) Secreted proteoglycans were separated by SDS-PAGE and the electrophoretic migration was graphically represented for (B) basal conditions, (C) PDGF conditions and (D) TGF-β1 conditions. Dotted lines indicate peak of biglycan and decorin from control and growth factor treated SMCs. Gel is representative of 1 experiment. To facilitate data analysis, scans were strategically positioned to reflect the order of appearance on the gel using Fig.P Version 2.98.

Treatment of vascular SMCs with G-ester (20 µmol/L) had no significant effect on [^{35}S]-sulfate incorporation into proteoglycans, in the presence and absence of PDGF or TGF-β1 (Figure 3-34. Human vascular SMCs treated with G-acid (20 µmol/L) showed no change in the incorporation of [^{35}S]-sulfate into proteoglycans, in the presence and absence of growth factors, PDGF and TGF-β1 (Figure 3-34. Treatment of vascular SMCs with G-amide (20 µmol/L) decreased [^{35}S]-sulfate incorporation into proteoglycans in the presence and absence of growth factors, by 26.2% ($P<0.01$) compared to untreated SMCs, by 29.0% ($P<0.001$) compared to cells treated with PDGF alone and by 11.8% (not significant) compared to cells treated with TGF-β1 alone (Figure 3-34). Analysis of the electrophoretic mobility of proteoglycans synthesized in the presence of G-ester (20 µmol/L) showed that there was no change in the mobility of these proteoglycans, whether the SMCs were exposed to G-ester in the presence or absence of growth factors, PDGF and TGF-β1 (Figure 3-35A, B, C and D). Proteoglycans from vascular SMCs treated with G-acid (20 µmol/L) in the presence and absence of growth factors, had an increased electrophoretic mobility by SDS-PAGE compared to the respective controls (Figure 3-35A, B, C and D). The proteoglycans from vascular SMCs treated with G-amide (20 µmol/L) and separated by electrophoresis, migrated in a similar manner to proteoglycans from SMCs maintained under basal conditions (Figure 3-35A and B). The reduction in sulfate incorporation into proteoglycans following the treatment of vascular SMCs with G-amide in the presence of PDGF or TGF-β1, was not associated with a change in the electrophoretic mobility by SDS-PAGE (Figure 3-35A, C and D).
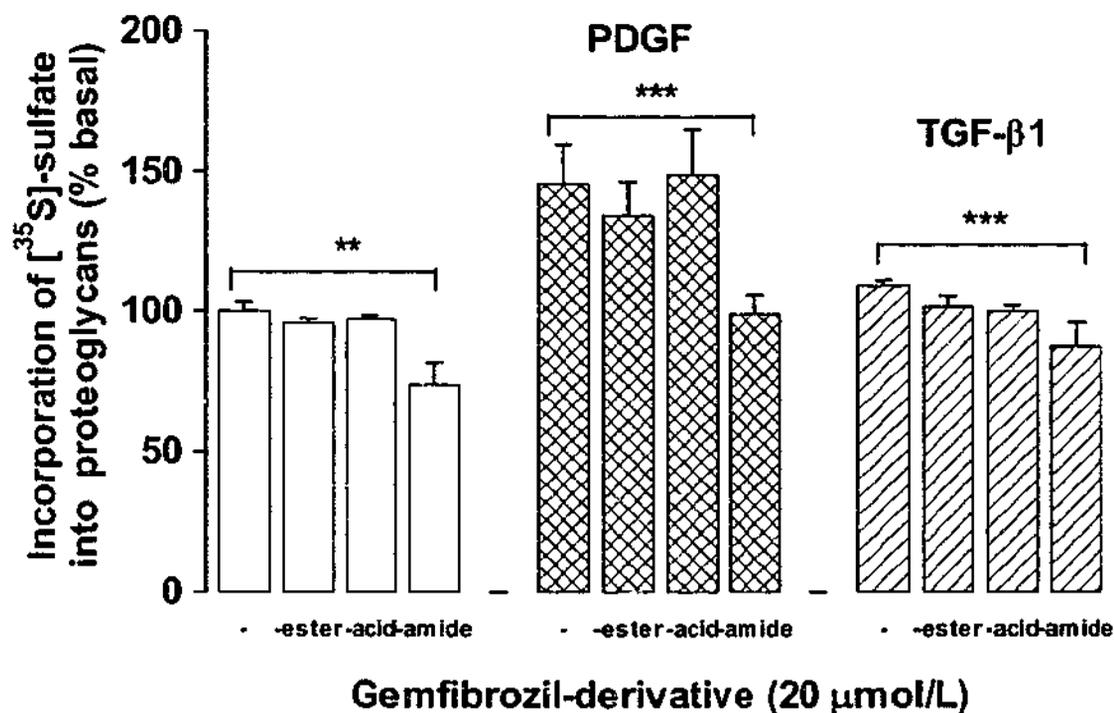
Figure 3-34

Gemfibrozil-amide reduces [^{35}S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with 0.1% DMSO (-) or gemfibrozil-ester, -acid or -amide (each at 20 μmol/L) in the presence and absence of TGF-β1 (1 ng/mL) or PDGF (50 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Incorporation of [^{35}S]-sulfate into proteoglycans was assessed by the CPC precipitation assay. Results are mean±SEM of 2 experiments performed in triplicate (** $P<0.01$, *** $P<0.001$). Pre-normalized basal value is 235.3±8.2 cpm [^{35}S]-sulfate/10^3 cells.
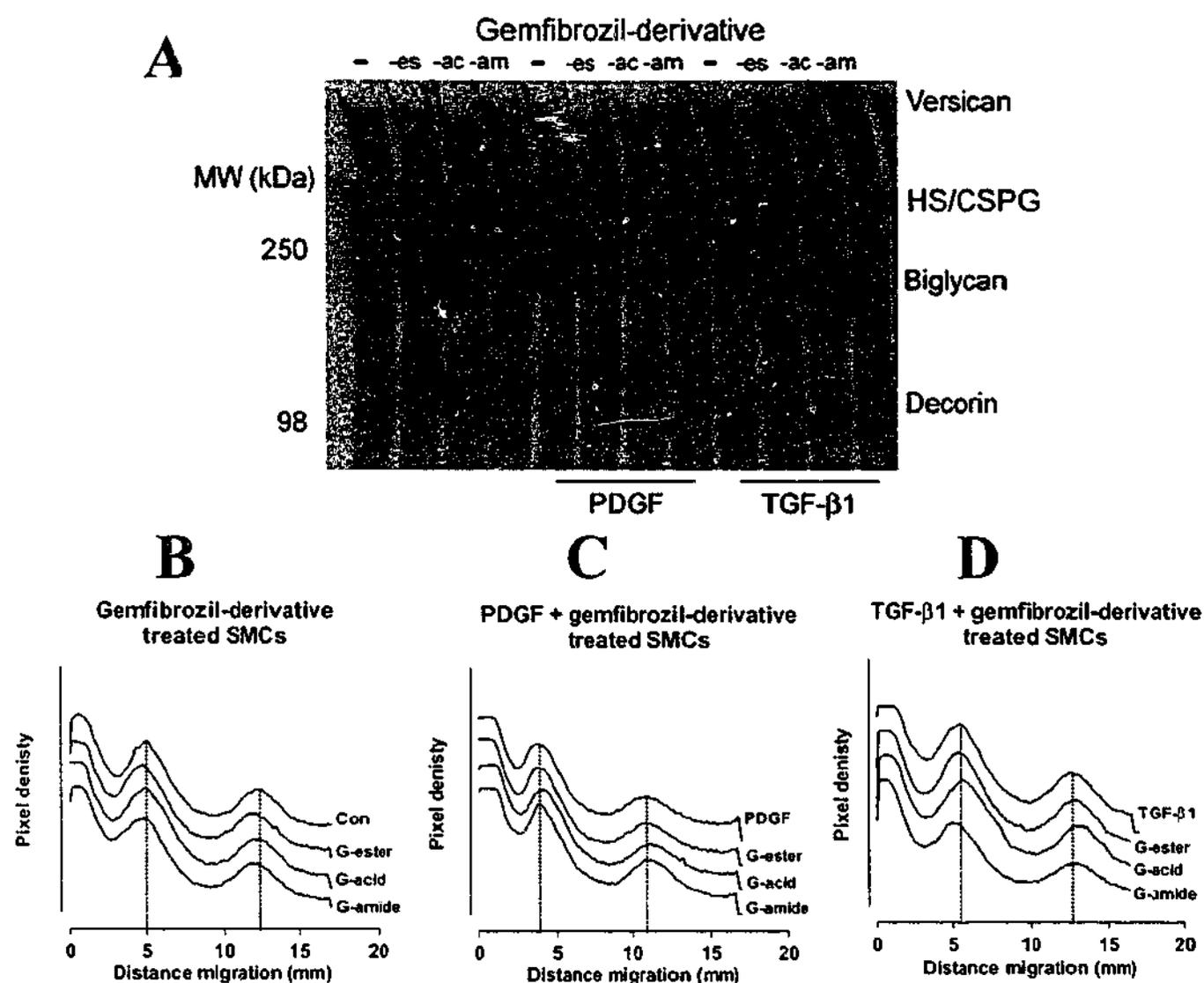
Figure 3-35

Gemfibrozil-acid treatment of human vascular SMCs increases the electrophoretic mobility of secreted proteoglycans. Human vascular SMCs were treated with 0.1% DMSO (- or Con) or gemfibrozil-ester (-es; G-ester), -acid (-ac; G-acid) or -amide (-am; G-amide), each at 20 μmol/L, in the presence and absence of TGF-β1 (1 ng/mL) or PDGF (50 ng/mL) and metabolically labelled with [^{35}S]-sulfate. (A) Secreted proteoglycans were separated by SDS-PAGE and the electrophoretic migration was graphically represented for (B) basal conditions, (C) PDGF conditions and (D) TGF-β1 conditions. Dotted lines indicate peak of biglycan and decorin from control lanes. Gel is representative of 1 experiment. To facilitate data analysis, scans were strategically positioned to reflect the order of appearance on the gel using Fig.P Version 2.98.

These studies have shown that treatment of vascular SMCs with the amide derivatives of fenofibrate and gemfibrozil, reduced sulfate incorporation into proteoglycans but did not alter GAG length. This suggests that the amide derivatives may have had a different molecular mechanism of action compared to fenofibrate or gemfibrozil. Overall, the findings were not consistent with our hypothesis that increased cell permeability rather than PPAR-α ligand activity could affect vascular SMC proteoglycan GAG length therefore, the study was not pursued further. Increased cell permeability may reduce sulfation of GAGs however, we were not in a position to follow this up as FACE was unavailable at this time.

3.2.14 A potent PPAR-α ligand, GW7647, has no significant effect on proteoglycan biosynthesis.

The studies presented thus far have shown that fenofibrate modifies the length of GAGs on proteoglycans however, it is unknown whether or not these effects are occurring through a PPAR-α-dependent or -independent mechanism. Fenofibrate is cleaved by esterases in the cell to release fenofibric acid, the active ligand for the PPAR-α. Fenofibric acid has a relatively low affinity for the PPAR-α with an EC_{50} value for the human receptor of 30 μmol/L (Brown et al., 2001; Fruchart et al., 2001). In an attempt to determine whether or not PPAR-α was involved in controlling proteoglycan synthesis and GAG length, a PPAR-α ligand which is 5,000 times more potent than fenofibric acid, GW7647, was assessed for effects on human vascular SMC proteoglycan synthesis and GAG length. The compound GW7647 is a urea substituted thioisobutyric acid and is not classified as a fibrate (Brown et al., 2001) (Refer to Chapter 1, Figure 1-2 for chemical structure).

To optimize any potential inhibitory effects of the nuclear receptor ligand, human vascular SMCs were pre-treated with GW7647 (0.01-1.00 μmol/L) for 6 h prior to stimulation with

growth factors. Following pre-incubation with GW7647, the growth factors TGF-β1 (2 ng/mL) or PDGF (20 ng/mL) with insulin (1 μmol/L) were added for 16 h, the latter combination being an *in vitro* model used by others (Wakino *et al.*, 2000) to mimic the atherogenic and hyperinsulinemic environment to which vessels are exposed in subjects with type 2 diabetes. Human vascular SMCs were metabolically labelled with [^{35}S]-sulfate following the addition of growth factors, for a further 24 hours. Fenofibrate (30 μmol/L) was included in the experiments under the conditions tested with GW7647, as a positive control for inhibition of proteoglycan synthesis and GAG elongation. Incorporation of [^{35}S]-sulfate into proteoglycans was quantitated by the CPC precipitation assay and the electrophoretic mobility of the proteoglycans was assessed by SDS-PAGE.

Human vascular SMCs treated with GW7647 (0.01-1.00 μmol/L) showed a weak concentration-related reduction in [^{35}S]-sulfate incorporation into proteoglycans however, treatment with fenofibrate (30 μmol/L) resulted in a 53.9% ($P<0.001$) decrease in [^{35}S]-sulfate incorporation into proteoglycans compared to untreated SMCs (Figure 3-36). Stimulation of vascular SMCs with TGF-β1 (2 ng/mL) significantly increased [^{35}S]-sulfate incorporation into proteoglycans from 154.1±28.5 cpm/10^3 cells under basal conditions to 240.5±45.0 cpm/10^3 cells in the presence of TGF-β1 (Figure 3-37). Treatment of vascular SMCs with GW7647 (0.01-1.00 μmol/L) in the presence of TGF-β1 showed a weak concentration-related reduction in [^{35}S]-sulfate incorporation into proteoglycans, however, treatment with fenofibrate had a significant effect to reduce [^{35}S]-sulfate incorporation into proteoglycans by 47.3% ($P<0.001$) compared to cells treated with TGF-β1 alone (Figure 3-37). Treatment of human vascular SMCs with GW7647 (0.01-1.00 μmol/L) in the presence of PDGF (20 ng/mL) with insulin (1 μmol/L) did not alter [^{35}S]-sulfate incorporation

into proteoglycans, however under these conditions the treatment of vascular SMCs with fenofibrate (30 μmol/L) reduced [^{35}S]-sulfate incorporation into proteoglycans by 32.8% ($P<0.001$; Figure 3-38).

Proteoglycans from SMCs treated with fenofibrate (30 μmol/L) showed an increase in the electrophoretic migration by SDS-PAGE compared to proteoglycans from untreated cells (Figure 3-39A and B). The treatment of human vascular SMCs with GW7647 (0.01-1.00 μmol/L) was associated with a concentration-related increase in the electrophoretic mobility of the proteoglycans by SDS-PAGE (Figure 3-39A and B). Proteoglycans from SMCs treated with fenofibrate in the presence of TGF-β1 had an increased mobility by SDS-PAGE compared to proteoglycans from SMCs stimulated with TGF-β1 alone (Figure 3-40A and B). Conversely, the electrophoretic mobility of proteoglycans synthesized by vascular SMCs in the presence of GW7647 and TGF-β1 did not change compared to cells treated with TGF-β1 alone (Figure 3-39A and B). Human SMCs treated with fenofibrate in the presence of PDGF/insulin, synthesized proteoglycans with an increased electrophoretic mobility compared to proteoglycans from SMCs treated with PDGF/insulin alone (Figure 3-40A and B). Treatment of vascular SMCs with GW7647 (0.01-1.00 μmol/L) in the presence of PDGF/insulin did not change the electrophoretic mobility of proteoglycans by SDS-PAGE compared to cells treated with PDGF/insulin alone (Figure 3-41A and B).

GW7647 concentration response
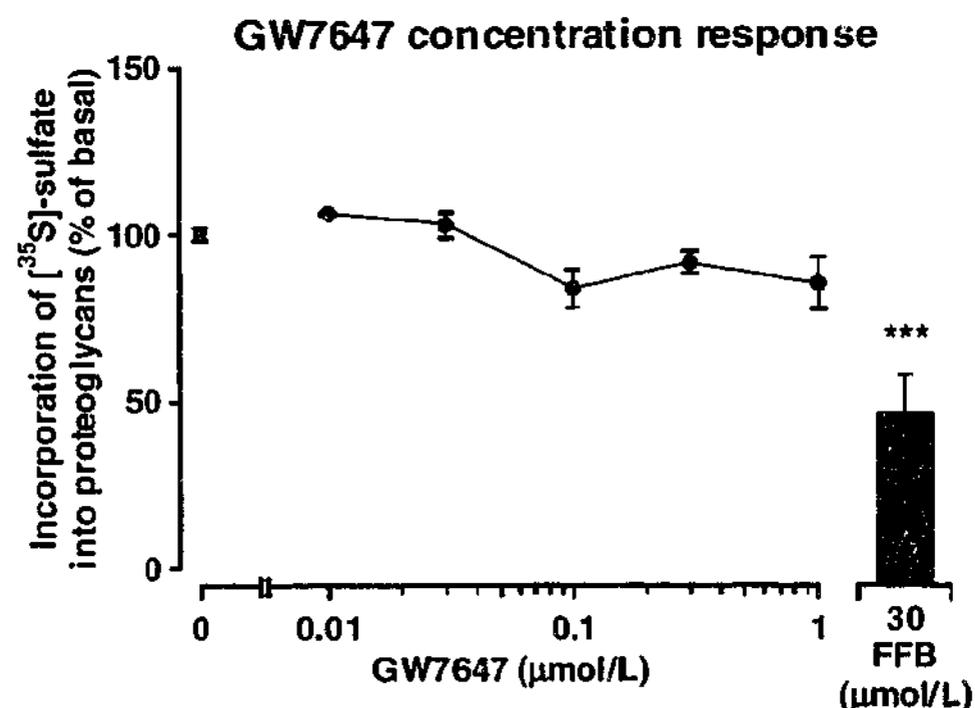
Figure 3-36

Treatment of human vascular SMCs with GW7647 does not alter [35S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with GW7647 (0.01-1 µmol/L) or fenofibrate (30 µmol/L; FFB) and metabolically labelled with [35S]-sulfate. Secreted proteoglycans were quantitated by the CPC precipitation assay. Data represent 3 experiments performed in triplicate (*** $P<0.001$ versus no drug). Pre-normalized basal value is 154.1±28.5 cpm [35S]-sulfate/10^3 cells.
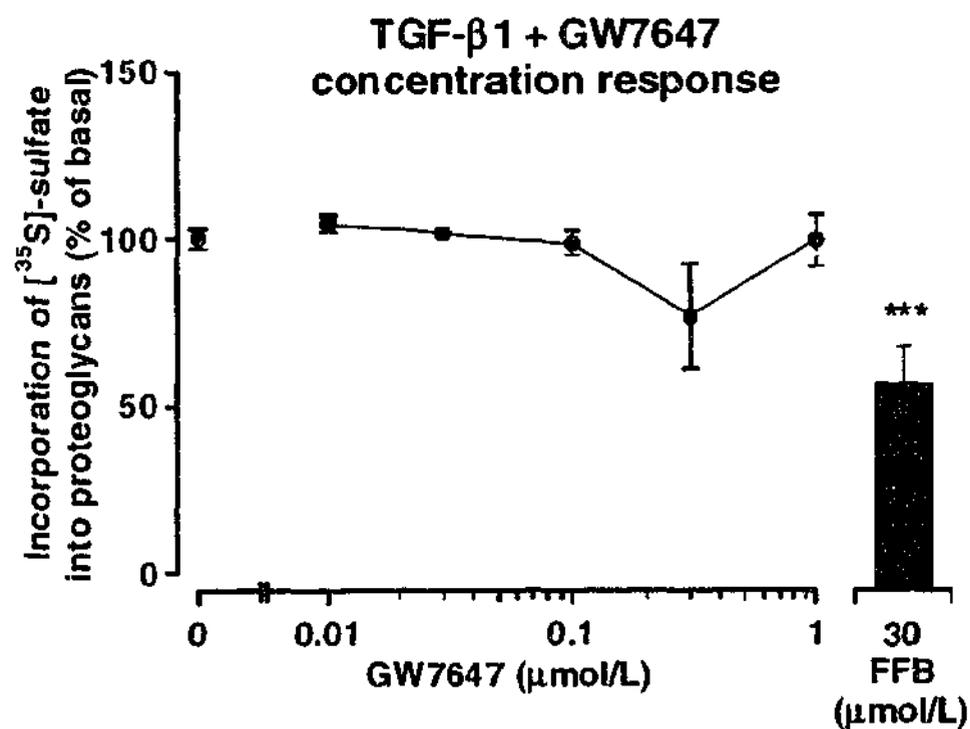
Figure 3-37

Treatment of human vascular SMCs with GW7647 in the presence of TGF-β1 does not alter [^{35}S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with GW7647 (0.01-1 μmol/L) or fenofibrate (30 μmol/L; FFB) in the presence of TGF-β1 (2 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Secreted proteoglycans were quantitated by the CPC precipitation assay. Data represent 3 experiments performed in triplicate (*** $P<0.001$ versus TGF-β1 alone). Pre-normalized TGF-β1 value is 240.5±45.0 cpm [^{35}S]-sulfate/10^3 cells.

PDGF + GW7647
concentration response
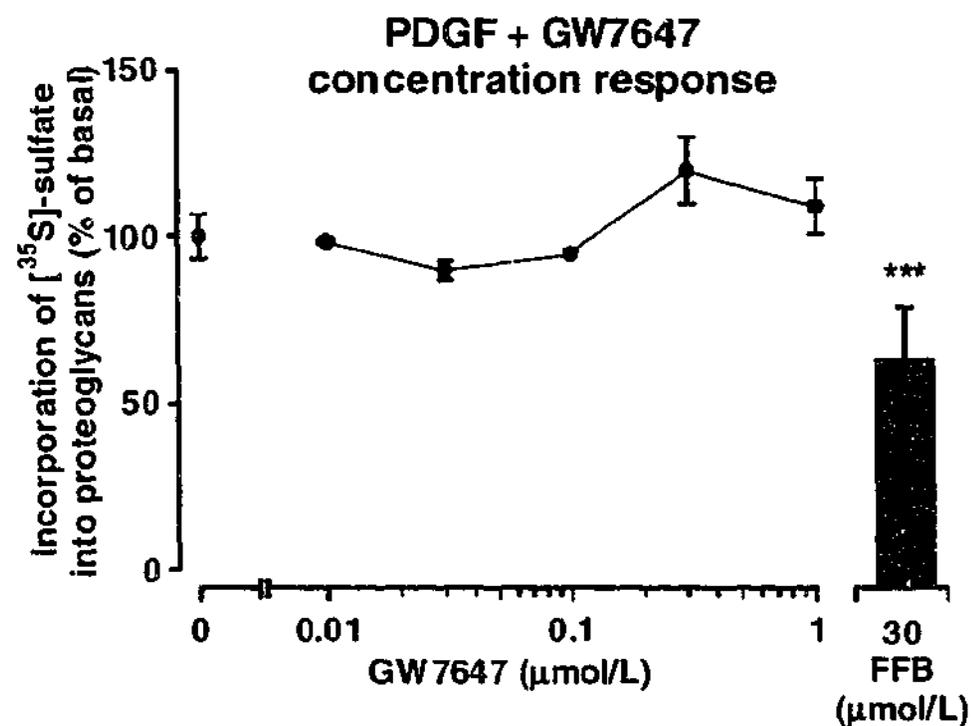
Figure 3-38

Treatment of human vascular SMCs with GW7647 in the presence of PDGF does not alter proteoglycan synthesis. Human vascular SMCs were treated with GW7647 (0.01-1 μmol/L) or fenofibrate (30 μmol/L; FFB) in the presence of PDGF (20 ng/mL) and metabolically labelled with $[^{35}S]$-sulfate. Secreted proteoglycans were quantitated by the CPC assay. Data represent 3 experiments performed in triplicate (*** $P<0.001$ versus PDGF alone). Pre-normalized PDGF value is 180.4 ± 36.8 cpm $[^{35}S]$-sulfate/10^3 cells.
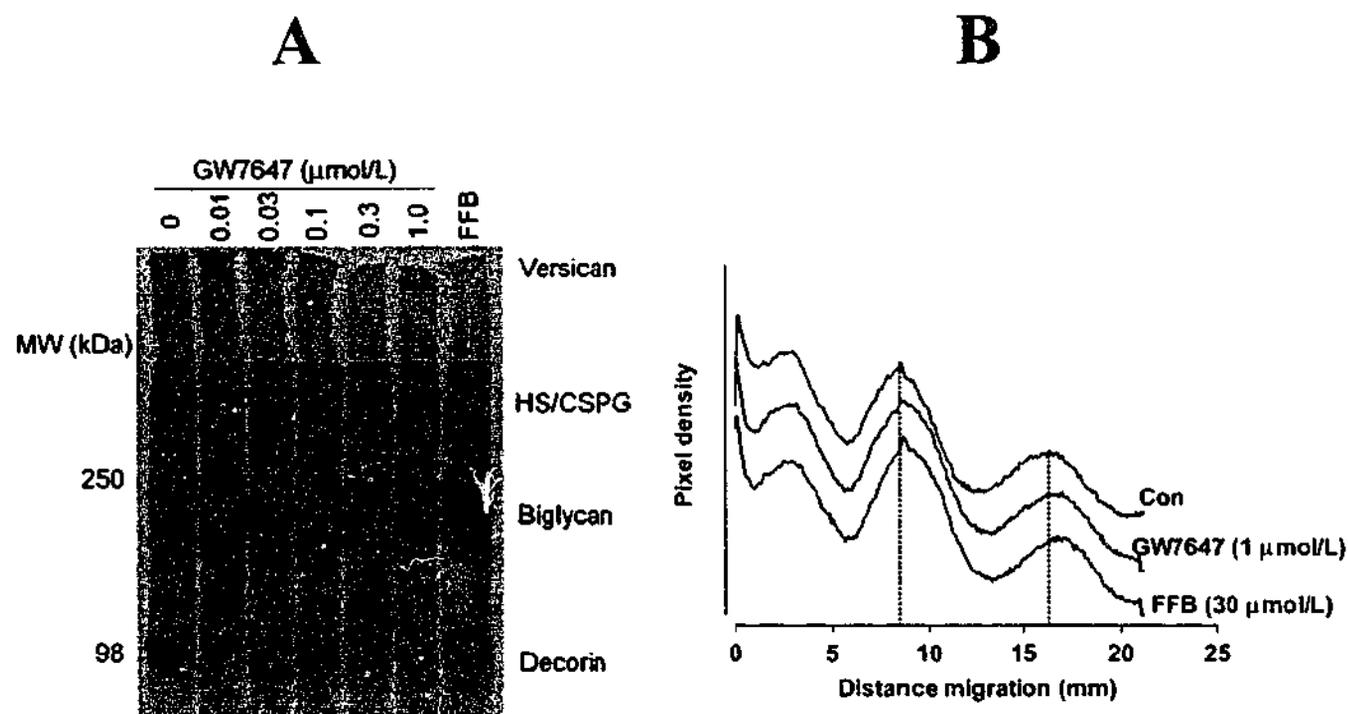
Figure 3-39

Human vascular SMCs treated with GW7647 show increased electrophoretic mobility by SDS-PAGE. Human vascular SMCs were treated with GW7647 (0.01-1 μmol/L) or fenofibrate (30 μmol/L; FFB) and metabolically labelled with [^{35}S]-sulfate. (A) Secreted proteoglycans were isolated from the culture medium and separated by SDS-PAGE. (B) Electrophoretic migration of proteoglycans in the resolving gel from A was graphically represented. Dashed lines indicate the peak of biglycan and decorin from untreated SMCs. Data represents similar results from 3 experiments. To facilitate data analysis, scans were strategically positioned to reflect the position on the gel using Fig.P Version 2.98.
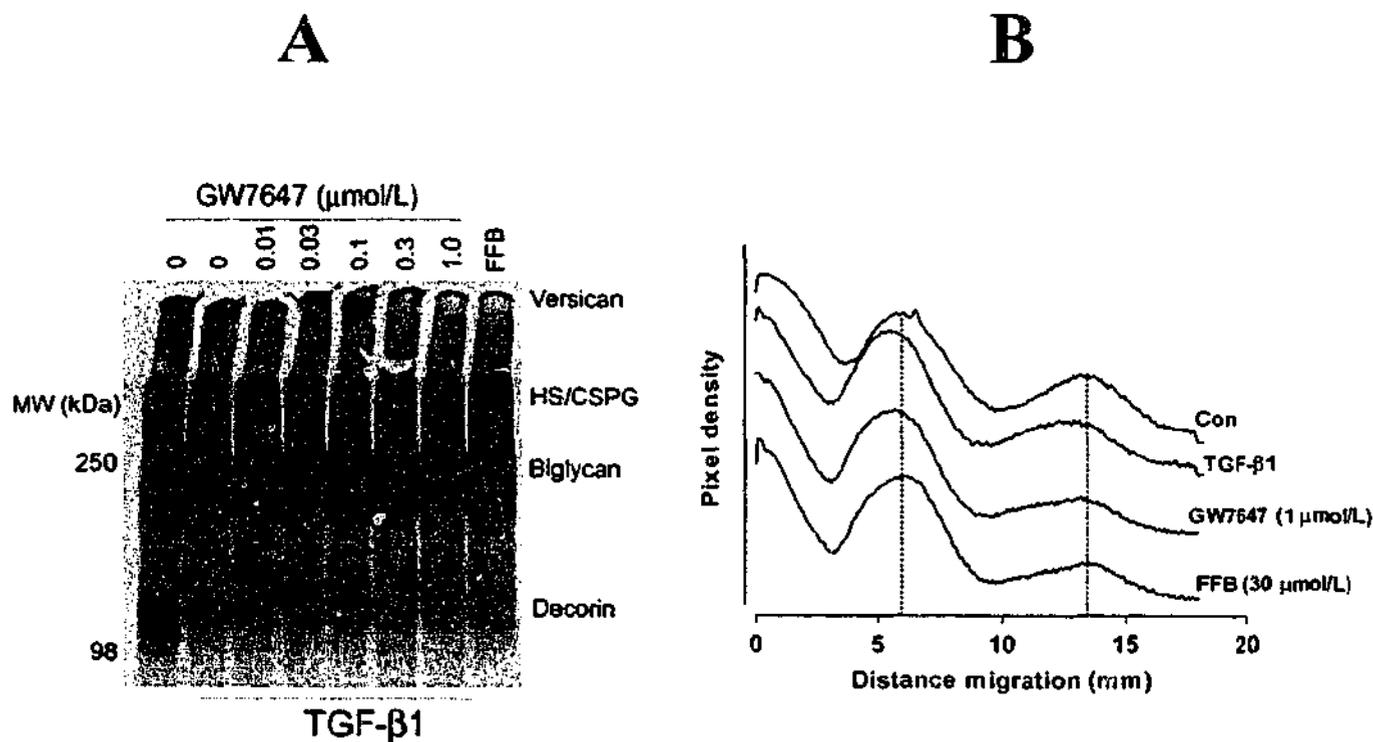
Figure 3-40

Treatment of human vascular SMCs with GW7647 in the presence of TGF-β1 does not alter the electrophoretic mobility of proteoglycans by SDS-PAGE. Human vascular SMCs were treated with GW7647 (0.01-1 µmol/L) or fenofibrate (30 µmol/L; FFB) in the presence of TGF-β1 (2 ng/mL) and metabolically labelled with [^{35}S]-sulfate. (A) Secreted proteoglycans were isolated from the culture medium and separated by SDS-PAGE. (B) Electrophoretic migration of proteoglycans in the resolving gel from A was graphically represented. Con is SMCs treated with 0.1% DMSO. Dashed lines indicate the peak of biglycan and decorin from untreated SMCs. Data represents similar results from 3 experiments. To facilitate data analysis, scans were strategically positioned to reflect the position on the gel using Fig.P Version 2.98.

A

GW7647 (µmol/L)

0   0   0.01   0.03   0.1   0.3   1.0   FFB

MW (kDa)

250

98

Versican

HS/CSPG

Biglycan

Decorin

PDGF + insulin

B

Pixel density

Con

PDGF/insulin

GW7647 (1 µmol/L)

FFB (30 µmol/L)

0   5   10   15   20

Distance migration (mm)

Figure 3-41

Treatment of human vascular SMCs with GW7647 in the presence of PDGF does not alter the electrophoretic mobility of proteoglycans by SDS-PAGE. Human vascular SMCs were treated with GW7647 (0.01-1 µmol/L) or fenofibrate (30 µmol/L; FFB) in the presence of PDGF (20 ng/mL)/insulin (1 µmol/L) and metabolically labelled with $[^{35}S]$-sulfate. (A) Secreted proteoglycans were isolated from the culture medium and separated by SDS-PAGE. (B) Electrophoretic migration of proteoglycans in the resolving gel from A was graphically represented. Con is SMCs treated with 0.1% DMSO. Dashed lines indicate the peak of biglycan and decorin from untreated SMCs. Data represents similar results from 3 experiments. To facilitate data analysis, scans were strategically positioned to reflect the position on the gel using Fig.P Version 2.98.

In summary, fenofibrate (30 μmol/L) treatment of SMCs in the presence and absence of growth factors, had a greater effect on reducing sulfate incorporation into proteoglycans than the highest concentration of GW7647 (1 μmol/L; Figures 3-36, 3-37, 3-38). Despite the higher potency for PPAR-α than fenofibrate, GW7647 treatment of human vascular SMCs in the presence of atherogenic growth factors did not reduce GAG length. It follows that fenofibrate, which has a lower potency for PPAR-α than GW7647, has a greater effect to reduce GAG length in the presence of atherogenic growth factors. These data support the results presented earlier in this study of the effect of fenofibrate to reduce GAG length (Section 3.2.7 to 3.2.9) however, the findings with GW7647 suggest that increased PPAR-α affinity does not affect proteoglycan synthesis or GAG length. The effect of GW7647 to induce a small reduction in GAG length in the absence of growth factors was not pursued.

3.2.15 The effect of combining a PPAR-α ligand with a PPAR-γ ligand on proteoglycan synthesis

The studies in the earlier parts of this chapter have shown that fibrates/PPAR-α ligands have direct vascular actions to reduce proteoglycan synthesis and modify their structure. PPAR-γ ligands such as troglitazone were shown in experiments evolving in our laboratory at this time, to also reduce GAG length. PPAR-α and -γ are regulators of many genes and may also act on other signalling pathways. Combined therapy of these agents occurs in subjects with type 2 diabetes such as the PPAR-γ ligand, a thiazolidinedione (e.g. rosiglitazone) to treat hyperglycemia and a PPAR-α ligand to treat the dyslipidemia of diabetes (e.g. fenofibrate). We addressed the question as to whether or not the vascular actions of a PPAR-γ ligand would block or enhance the inhibitory effect of GAG elongation demonstrated for fibrates/PPAR-α

ligands. We chose GW7647 in the expectation of getting a potent PPAR-α ligand at a very low concentration such that combining drug treatments could be done with minimal toxicity however, it is noted that GW7647 treatment of SMCs was not as efficacious as fenofibrate in GAG shortening (Section 3.2.14). Notwithstanding these limitations, the question of PPAR-α/-γ interactions was considered to be sufficiently important to address experimentally.

Confluent cultures of human vascular SMCs were pre-treated with GW7647 (1 μmol/L) and troglitazone (10 μmol/L) for 6 hours followed by stimulation with TGF-β1 (2 ng/mL). Vascular SMCs were metabolically labelled with [^{35}S]-sulfate and secreted proteoglycans were quantitated by the CPC precipitation assay and assessed for changes in the electrophoretic mobility by SDS-PAGE.

The treatment of vascular SMCs with GW7647 (1 μmol/L), troglitazone (10 μmol/L) and the combination of the two agents under basal conditions did not have an effect on [^{35}S]-sulfate incorporation into proteoglycans (Figure 3-42). Treatment of human vascular SMCs with TGF-β1 increased proteoglycan synthesis 2-fold compared to untreated cells (Figure 3-42). The treatment of vascular SMCs with GW7647 or troglitazone in the presence of TGF-β1, decreased proteoglycan synthesis by 22.0% and 13.8% respectively, compared to cells treated with TGF-β1 alone (Figure 3-42). The combination of GW7647 and troglitazone in the presence of TGF-β1 resulted in less inhibition (8.8%) of proteoglycan synthesis than with either PPAR ligand alone (Figure 3-42). Biglycan synthesized in the presence of GW7647 or troglitazone alone showed an increase in the electrophoretic mobility by SDS-PAGE, compared to biglycan from cells maintained under basal conditions (Figure 3-42A and B). The treatment of vascular SMCs with both GW7647 and troglitazone did not change the electrophoretic mobility of the biglycan synthesized compared to proteoglycans from untreated cells (Figure 3-43A and B). Stimulation of vascular SMCs with TGF-β1,

reduced the electrophoretic mobility of biglycan compared to proteoglycans from untreated cells (Figure 3-43A). In the presence of TGF-β1, treatment of vascular SMCs with troglitazone increased the electrophoretic mobility of biglycan compared to cells treated with TGF-β1 alone (Figure 3-43A and C). Vascular SMCs treated with both GW7647 and troglitazone in the presence of TGF-β1 showed no additive effect on the electrophoretic mobility of biglycan compared to proteoglycans from cells treated with TGF-β1 alone (Figure 3-43A and C). However, the increase in electrophoretic mobility of biglycan from troglitazone treated SMCs is not prevented by combining troglitazone with GW7647 (Figure 3-43A and C).

In summary, these data indicate that the combination of a PPAR-α ligand with a PPAR-γ ligand does not alter the incorporation of sulfate into proteoglycans or modify GAG length. The limited number of experiments conducted in this study and the complexity of combining agents which each have multiple actions in vascular SMCs, make it difficult to conclude whether or not there is additional benefit of combining these agents on vascular proteoglycans *in vitro*. The potentially beneficial effects of troglitazone on vascular proteoglycans does not appear to be regulated by the potent PPAR-α ligand (see Figure 3-43 A and C). Further experiments are required to make any conclusions on this aspect of PPAR-α/-γ combined regulation of proteoglycan synthesis and structure.
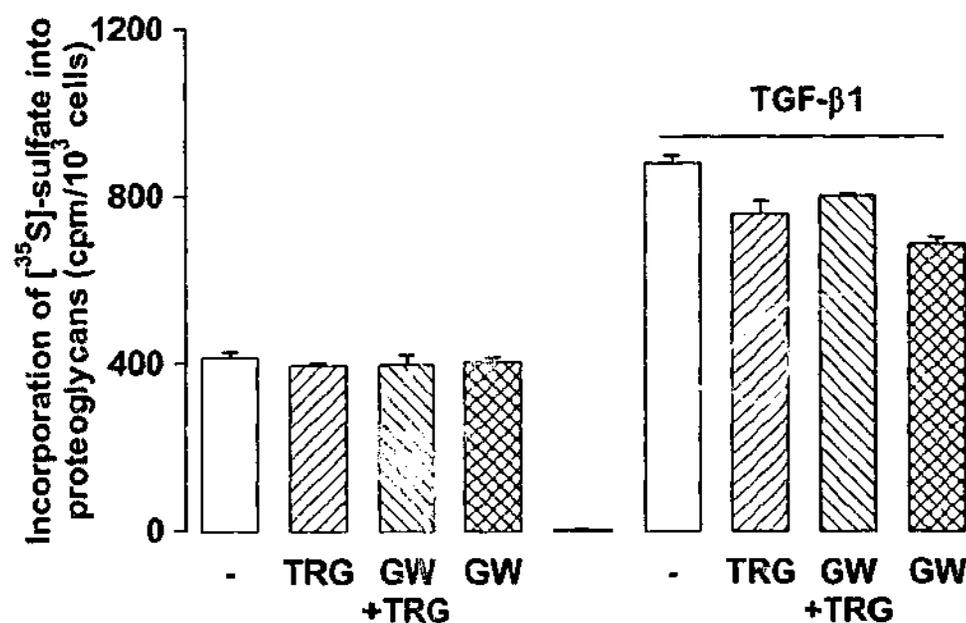
Figure 3-42

The combination of a PPAR-α ligand and a PPAR-γ ligand is not additive on reducing [³⁵S]-sulfate incorporation into proteoglycans. Human vascular SMCs were treated with 0.1% DMSO (-), troglitazone (TRG; 10 µmol/L), GW7647 (GW; 1 µmol/L), or the combination of GW7647 with troglitazone, in the presence and absence of TGF-β1 (2 ng/mL). Vascular SMCs were metabolically labelled [³⁵S]-sulfate and secreted proteoglycans were quantitated by the CPC precipitation assay. Data represents 1 experiment performed in triplicate.
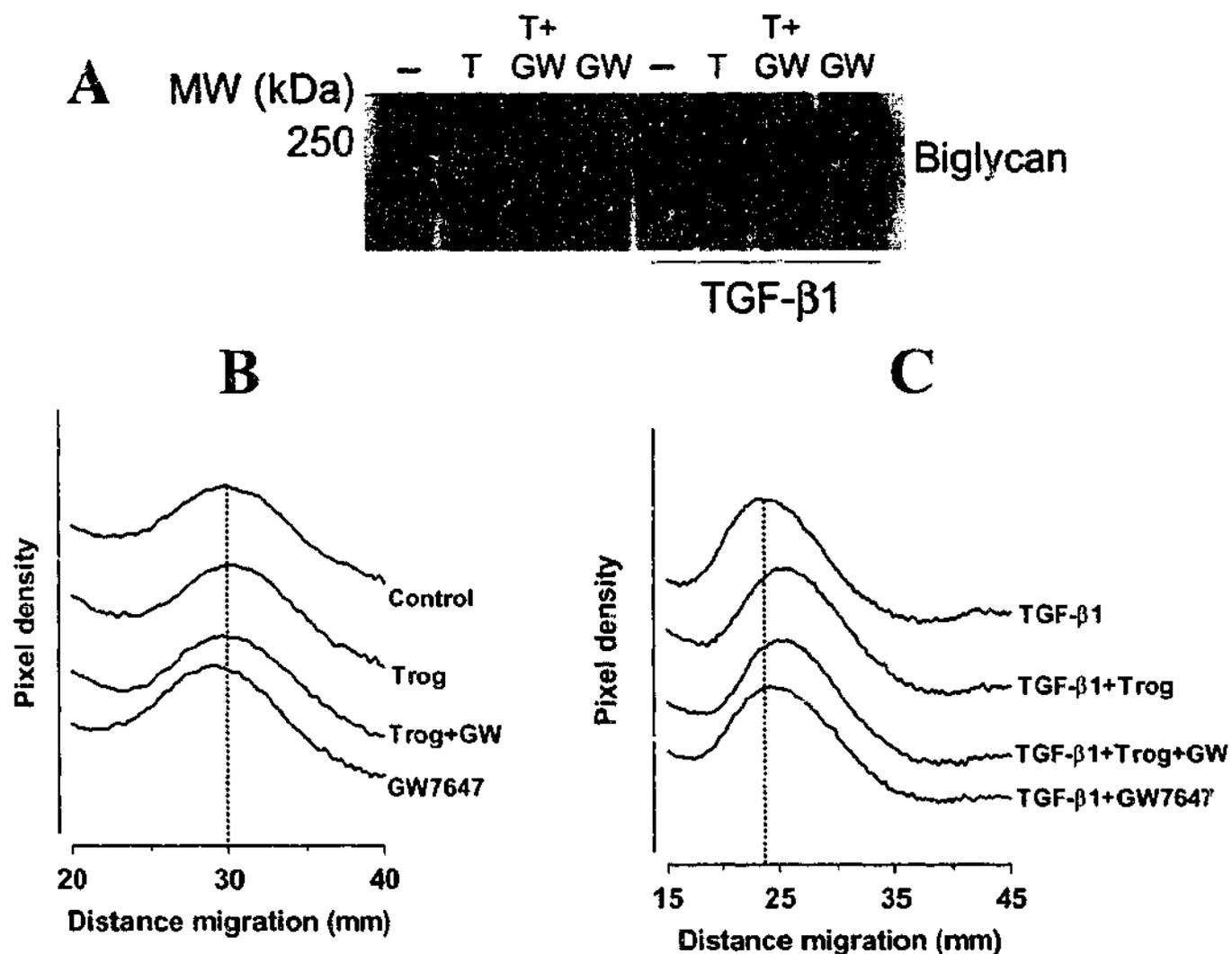
Figure 3-43

The combined treatment of SMCs with a PPAR-α ligand and a PPAR-γ ligand is not additive on increasing the electrophoretic mobility of biglycan. Human vascular SMCs were treated with 0.1% DMSO (-), troglitazone (T; 10 μmol/L) alone, GW7647 (GW; 1 μmol/L) alone, or the combination of GW7647 with troglitazone, in the presence and absence of TGF-β1 (2 ng/mL). (A) Secreted proteoglycans were separated on a 4-13% linear gradient SDS-PAGE. (B and C) The electrophoretic migration of biglycan from A was graphically represented. Dashed lines indicate the peak of biglycan from (B) untreated or (C) TGF-β1 treated SMCs. Data represents results from 1 experiment. To facilitate data analysis, scans were strategically positioned to reflect the position on the gel using Fig.P Version 2.98.

3.3   Summary of results for Chapter 3

The studies presented in this chapter have shown that the effect of fenofibrate and other various PPAR-α ligands on vascular SMCs is a modification of the secretion and structure of proteoglycans. A summary of the findings from these studies include:

- The *in vitro* model used for the analysis of proteoglycans contained positively identified SMCs which showed expression of PPAR-α, the receptor for which fenofibrate is a ligand;

- In human vascular SMCs, concentrations of fenofibrate above 50 μmol/L inhibit normal protein synthesis, and may be taken as an indication of toxicity;

- Fibrate (fenofibrate, fenofibric acid and gemfibrozil) treatment of vascular SMCs increased total proteoglycan core protein synthesis;

- Fenofibrate and fenofibric acid treatment of vascular SMCs in the presence and absence of atherogenic growth factors, TGF-β1 and PDGF, induces concentration-related reductions in [^{35}S]-sulfate incorporation into proteoglycans;

- Treatment of vascular SMC with fenofibrate in the presence and absence of growth factors, increased [^{3}H]-glucosamine incorporation into glycosaminoglycans;

- Proteoglycans from vascular SMCs treated with fenofibrate or fenofibric acid in the presence and absence of growth factors show a concentration-related increase in the electrophoretic mobility by SDS-PAGE;

- Fenofibrate treatment of human SMCs supplemented with media containing xyloside, showed reduced synthesis of secreted free chains and these chains showed increased electrophoretic mobility by SDS-PAGE;

- Fenofibrate treatment of vascular SMCs in the presence of TGF-β1, showed small but consistent reductions in GAG length of intact proteoglycans, cleaved GAG chains and secreted free chains, when analyzed for size by gel filtration chromatography;

- Fenofibrate treatment of human vascular SMCs in the presence and absence of TGF-β1 does not alter the sulfation pattern of glycosaminoglycans;

- Digestion of proteoglycan GAG chains and subsequent analysis by SDS-PAGE showed that fenofibrate treatment of vascular SMCs may reduce dermatan sulfate GAGs on versican and decorin;

- The reduction in sulfate incorporation into proteoglycans following treatment of vascular SMCs with fenofibrate occurred in a time-dependent manner and was partially reversible in the presence of TGF-β1;

- Treatment of vascular SMCs with fenofibrate/gemfibrozil-amide which have similar cell permeability properties to fenofibrate/gemfibrozil-ester but lower PPAR-α activity, reduced sulfate incorporation into proteoglycans however this was not related to a an increase in the electrophoretic mobility of proteoglycans by SDS-PAGE;

- Treatment of vascular SMCs with the potent PPAR-α ligand, GW7647, did not alter proteoglycan synthesis nor modify the migration of proteoglycans by SDS-PAGE;

- Treatment of vascular SMCs with the combination of a PPAR-γ with a PPAR-α ligand did not have an additive effect to reduce GAG length.

3.4 Discussion of results for Chapter 3

Fenofibrate and fenofibric acid treatment of vascular SMCs in the presence and absence of atherogenic growth factors, TGF-β1 and PDGF, induced concentration-related reductions in proteoglycan synthesis. The reductions in sulfate incorporation were associated with increased electrophoretic mobility by SDS-PAGE, indicating a reduction in GAG length. To demonstrate that the effects of fenofibrate treatment specifically altered the GAG synthesizing mechanisms, xyloside supplemented SMCs were treated with fenofibrate and this also resulted in reduced synthesis and increased electrophoretic mobility of secreted xyloside-initiated GAGs (secreted free chains). More definitive analysis of size by gel filtration confirmed that the treatment of SMCs with fenofibrate in the presence of TGF-β1, reduced the length of GAG chains on proteoglycans and the secreted free chains.

The treatment of vascular SMCs with fenofibrate does not alter the sulfation pattern of GAGs as assessed by FACE however, the composition of the GAGs is altered. The characterization of GAG composition by using various GAG digestion enzymes and separation of undigested material using SDS-PAGE showed that human vascular SMCs synthesize versican, two large HS/CS proteoglycans, biglycan and decorin. The treatment of vascular SMCs with fenofibrate showed a reduction in the dermatan sulfate content of decorin suggesting that the level of the C-5-epimerase, which isomerizes glucuronic acid in CS GAGs to iduronic acid in DS GAGs, is less active under these conditions. Fenofibrate treatment of human vascular SMCs showed a reduction in the HS component and an increase in the CS component of the two unidentified HS/CS bands (bands 2 and 3) on SDS-PAGE. The effect of fenofibrate treatment to apparently increase KS GAGs may be due to aberrant keratanase activity.

The effect of fenofibrate treatment on vascular SMCs proteoglycan synthesis and GAG

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length occurred in time-dependent manner, however the effects were not permanent and could be reversed or partially reversed under growth factor conditions, when fenofibrate treatment was removed.

We hypothesized that the cell permeability of fenofibrate rather than the PPAR-α activity was responsible for the reductions in sulfate incorporation and GAG length of proteoglycans. The treatment of vascular SMCs with permeable fibrate derivatives, which we assumed had minimal PPAR-α activity (fenofibrate-amide and gemfibrozil-amide), in the presence and absence of growth factors, lead to a reduction in sulfate incorporation into proteoglycans however, this was not associated with a reduction in the length of GAG chains on proteoglycans. These data did not support the hypothesis that increased cell permeability rather than PPAR-α ligand activity is involved in controlling GAG length. To assess whether the effects of fenofibrate could be established with a more potent PPAR-α ligand, human vascular SMCs were treated with GW7647. Treatment of human vascular SMCs with GW7647 caused an increase in the electrophoretic mobility of secreted proteoglycans however unlike fenofibrate treatment, this was not observed in the presence of TGF-β1 or PDGF. These data are inconclusive as to whether or not the regulation of GAG length is dependent or independent of PPAR-α.

To complete the analysis of GAG synthesis the effects of fenofibrate on the incorporation of radiolabelled glucosamine was assessed. Fenofibrate treatment of human vascular SMCs in the presence and absence of atherogenic growth factors, unexpectedly increased the incorporation of [^{3}H]-glucosamine into secreted GAGs. An investigation of these anomalous results was undertaken and is presented in Chapter 5.

The treatment of vascular SMCs with fenofibrate induced changes in proteoglycan GAG length and composition which may relate to a perturbation of multiple cellular functions

important in atherogenesis (Wight, 1989). Of the many functions and roles that proteoglycans have in the vasculature related to atherogenesis such as cell proliferation (Kinsella & Wight, 1986; Shanahan *et al.*, 1997), we chose to assess whether or not the treatment of vascular SMCs with fenofibrate altered proteoglycan binding to LDL and this is presented in Chapter 4.

Chapter 4: Fibrate treatment of human vascular SMCs alters proteoglycans and the binding to LDL

4.1   Introduction to results of Chapter 4

This chapter is focused directly on the "response-to-retention" hypothesis of atherogenesis and specifically the binding of proteoglycans to LDL. Previous work by our group and others has shown that factors which increase the length of GAGs on vascular proteoglycans such as cell proliferation (Camejo *et al.*, 1993), TGF-β1 (Little *et al.*, 2002), oxidized-LDL (Chang *et al.*, 2000) and free fatty acids (Olsson *et al.*, 1999) results in increased binding to LDL. There are fewer examples of factors which reduce GAG length and binding to LDL, and these include the nutrient, glucosamine (Tannock *et al.*, 2002a) and calcium channel antagonists which are anti-hypertensives (Vijayagopal & Subramaniam, 2001). We have taken the approach that fibrates modulate proteoglycan synthesis including a reduction in GAG length and altered carbohydrate composition as presented in Chapter 3, which may result in reduced binding to LDL. The gel mobility shift assay (GMSA) has been optimized, as suggested by others (Hurt-Camejo *et al.*, 1998), for the proteoglycans from human vascular SMCs. The GMSA was used to assess the changes in binding of proteoglycans to LDL following treatment of vascular SMCs with growth factors and fibrates, gemfibrozil and fenofibrate. We have also used the GMSA to evaluate a different therapeutic intervention relating to the "response-to-retention" hypothesis of atherogenesis. This relates to a recent proposal that molecules such as "GAG mimetics" may interfere with the proteoglycan-LDL complex and reduce binding to LDL *in vivo* (Staels, 2002).

4.2 Results of Chapter 4

4.2.1 Optimization of the gel mobility assay

There are several assays that assess the binding of proteoglycans to LDL such as LDL affinity columns (Chang et al., 2000; Figueroa & Vijayagopal, 2002), interactions with unlabelled and labelled matrix (Sakr et al., 2001), competition studies of heparin bound LDL (Gigli et al., 1993) and binding of $[^{125}I]$-LDL to matrix of drug treated cells (Olsson et al., 1999) however, we chose to use the gel mobility shift assay (GMSA) (Hurt-Camejo et al., 1998). The GMSA involves the incubation of a fixed amount of proteoglycans with increasing concentrations of LDL and the separation of bound from unbound on a flatbed agarose gel. Although the gel mobility shift assay provides advantages over other assays such as simplicity, and the use of only a small amount of proteoglycan material, there are some important technical aspects of the design of the assay that should be considered. Changes in proteoglycan binding to LDL arise from changes in GAG length (Little et al., 2002), GAG charge density or sulfation pattern (Figueroa & Vijayagopal, 2002) or changes in GAG composition (Camejo et al., 1993; Tao et al., 1997). If the site of change (GAGs) are labelled with either $[^{35}S]$-sulfate or $[^3H]$-glucosamine for use in this assay, this may introduce confounding errors in the analysis. This is because the proteoglycans from different treatments are prepared and assessed by equal radioactivity however, when this is performed using GAG radiolabels, equal radioactivity will not necessarily represent equal amount of proteoglycan. We have recently suggested that using a core protein label such as $[^{35}S]$-methionine/cysteine or other radiolabelled amino acids is preferable (Ballinger et al., 2004) to a GAG chain radio-label. In doing so, we have assumed that while a particular treatment may alter the amount of core protein synthesized it will not change the physical properties of the core protein, such as size. Additionally, our laboratory has reported that LDL does not bind to

proteoglycan core proteins expressed in vascular SMC preparations which have had their GAG chains removed by enzymatic digestion as assessed by the GMSA (Little *et al.*, 2002; Tannock *et al.*, 2002a).

Another important consideration of the GMSA is the resultant data. A fixed amount of proteoglycans bound to a range of LDL concentrations (5-500 μg/mL) gives rise to binding curves which can be modelled to achieve half-maximal saturation values (Ballinger *et al.*, 2004) (Figure 4-1). Unlike classical drug-receptor binding interactions, the binding of proteoglycans to LDL is not reversible, and this should be considered when analyzing and interpreting data (Ballinger *et al.*, 2004). The data in this thesis are referred to as half-maximal saturation values with greater numbers indicating that higher amounts of LDL are required for saturation and represent lower "affinity" binding and *vice versa* (Ballinger *et al.*, 2004). The binding of increasing amounts of proteoglycans (250-2000 cpm) is saturable at 1000 cpm radiolabelled-proteoglycans with 500 μg/mL LDL but not 10 μg/mL LDL, indicating that proteoglycan-LDL binding is saturable in terms of proteoglycan concentration (Figure 4-2). These data show that the extent of binding is dependent upon both the concentration of LDL and the amount of proteoglycans.

A

LDL (µg/mL)

0   5   10   25   50   100   250   500
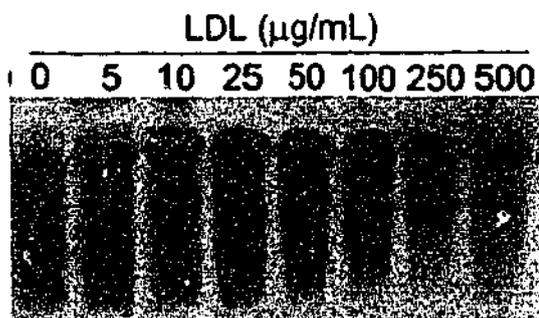
B
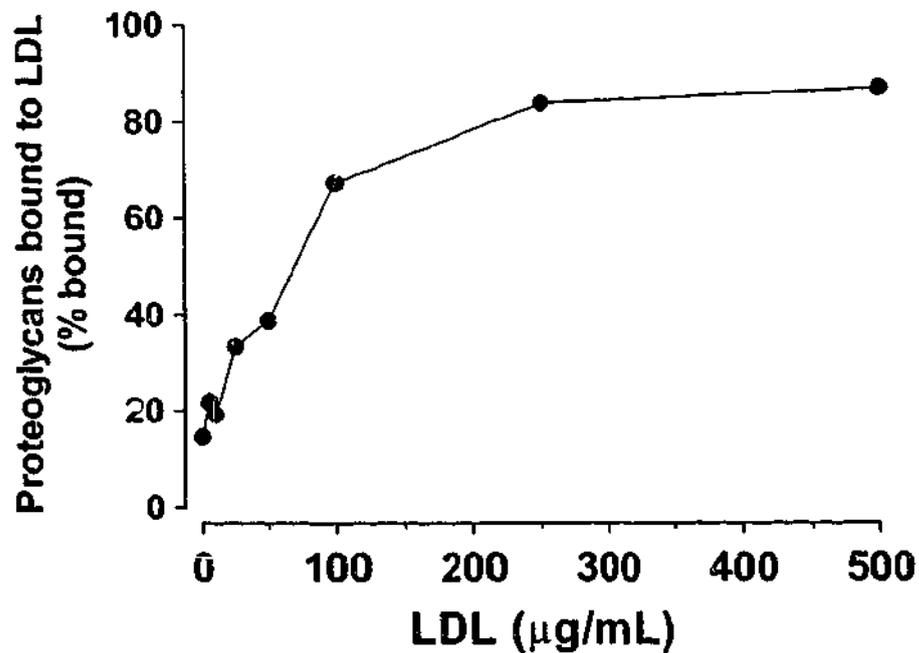
Figure 4-1

Proteoglycan LDL binding is saturable in terms of LDL concentration. Various amounts

of LDL (5-500 µg/mL) were incubated with a fixed amount of proteoglycans (1500 cpm). (A)

Bound and free proteoglycans were separated using the gel mobility shift assay and (B) the

percent of proteoglycans bound was plotted. These data represent one experiment and have

been published previously by our group (Ballinger *et al.*, 2004).

A

10 µg/mL   500 µg/mL

Proteoglycans (cpm)   Proteoglycans (cpm)

250   500   1000   2000   250   500   1000   2000
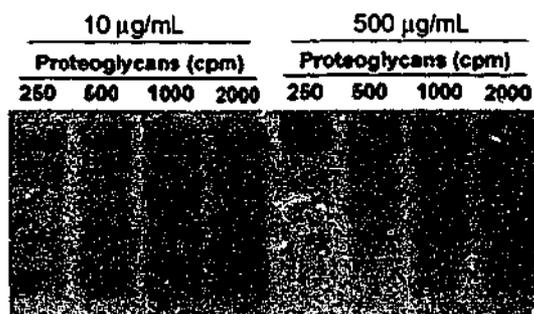
B
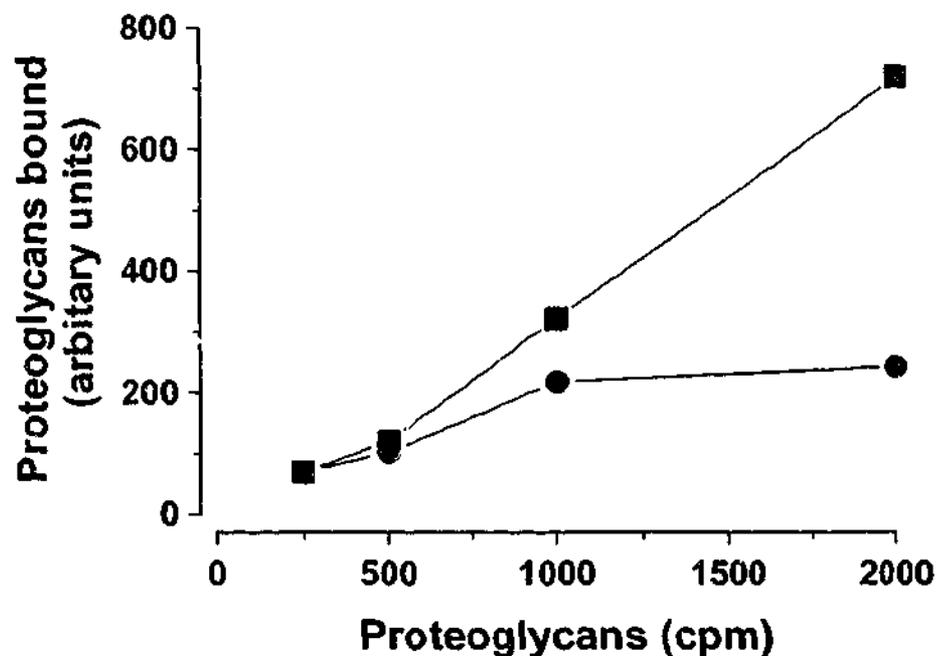
Figure 4-2

Proteoglycan:LDL binding is saturable in terms of proteoglycan concentration. Various

amounts of proteoglycans (250 – 2,000 cpm) were incubated with LDL at either 10 µg/mL

(closed circles) or 500 µg/mL (closed squares) LDL. (A) Bound and free proteoglycans were

separated using the gel mobility shift assay and (B) the density of the bound proteoglycans

was plotted. These data represent one experiment and have been published previously by our

group (Ballinger *et al.*, 2004).

4.2.2 Vascular SMCs treated with fenofibrate or gemfibrozil synthesize proteoglycans that show reduced binding to LDL

Substances which increase GAG length have been shown to contribute to an increase in LDL binding. For example, SMCs treated with TGF-β1 (Little *et al.*, 2002), angiotensin II (Figueroa & Vijayagopal, 2002) or oxidized-LDL (Chang *et al.*, 2000), synthesize proteoglycans with increased GAG length and these proteoglycans bind more to LDL than proteoglycans from untreated cells. Conversely, factors which reduce GAG length lead to reduced binding of proteoglycans to LDL. For example, SMCs treated with glucosamine (Tannock *et al.*, 2002a) or calcium channel antagonists (Vijayagopal & Subramaniam, 2001), synthesize proteoglycans with reduced GAG length and these proteoglycans bind less to LDL than proteoglycans from untreated cells. In the studies presented thus far, the treatment of vascular SMCs with fenofibrate in the presence and absence of growth factors results in the synthesis of proteoglycans with reduced GAG length and altered GAG composition. The reductions in GAG length are supported by similar observations made following the treatment of vascular SMCs with gemfibrozil, another clinically used fibrate/PPAR-α ligand (Nigro *et al.*, 2002). To determine whether the changes in proteoglycan GAG length and composition following treatment of vascular SMCs with fenofibrate (as described in Chapter 3, Section 3.2.7-3.2.9 and 3.2.11) or gemfibrozil (Nigro *et al.*, 2002), would modify their affinity f r LDL, we used the gel mobility shift assay (Hurt-Camejo *et al.*, 1998; Little *et al.*, 2002; Tannock *et al.*, 2002a). Vascular SMCs were incubated with [^{35}S]-methionine/cysteine to metabolically label proteoglycan core proteins. This labelling of core proteins (as opposed to [^{35}S]-sulfate labelling of GAGs) eliminated potentially confounding effects from having the radiolabel within the site of structural change (Ballinger *et al.*, 2004) as discussed in Section 4.2.1. Vascular SMCs were treated with either fenofibrate (30 μmol/L) or gemfibrozil

(100 µmol/L) in the presence and absence of growth factors, TGF-β1 (2 ng/mL) and PDGF (50 ng/mL) plus insulin (1 µmol/L).

The binding curve of proteoglycans to LDL from vascular SMCs treated with TGF-β1, was significantly different ($P<0.001$) from that for proteoglycans secreted from cells maintained under basal conditions (Figure 4-3). Stimulation of vascular smooth muscle cells with TGF-β1 resulted in the synthesis of proteoglycans that showed increased binding to LDL with a half maximal saturation concentration of 24.9±4.6 µg/mL LDL compared to 62.1±12.5 µg/mL LDL ($P<0.05$) for proteoglycans from untreated cells (Figure 4-3A and B). Treatment of vascular SMCs with PDGF results in the synthesis of proteoglycans with increased GAG length (Schonherr *et al.*, 1993; Schonherr *et al.*, 1991) compared to untreated cells however, it has not been demonstrated whether or not these proteoglycans show increased binding to LDL. We used PDGF with insulin as a model used by others (Wakino *et al.*, 2000), for the atherogenic and hyperinsulinemic environment to which blood vessels are exposed in subjects with type 2 diabetes (Chait & Bierman, 1994). The binding curve of vascular proteoglycans, from vascular SMCs treated with PDGF/insulin was significantly different ($P<0.001$) from that for proteoglycans from untreated cells (Figure 4-4A and B). Vascular SMCs stimulated with PDGF/insulin synthesize proteoglycans with a half-maximal saturation concentration of 9.5±4.4 µg/mL LDL compared to 27.9±3.2 µg/mL LDL ($P<0.05$) for proteoglycans synthesized under basal conditions.

A

Control

LDL (µg/mL)

0 5 10 25 50 100 250 500

Bound PG

Free PG

TGF-β1

LDL (µg/mL)

0 5 10 25 50 100 250 500

Bound PG

Free PG

B
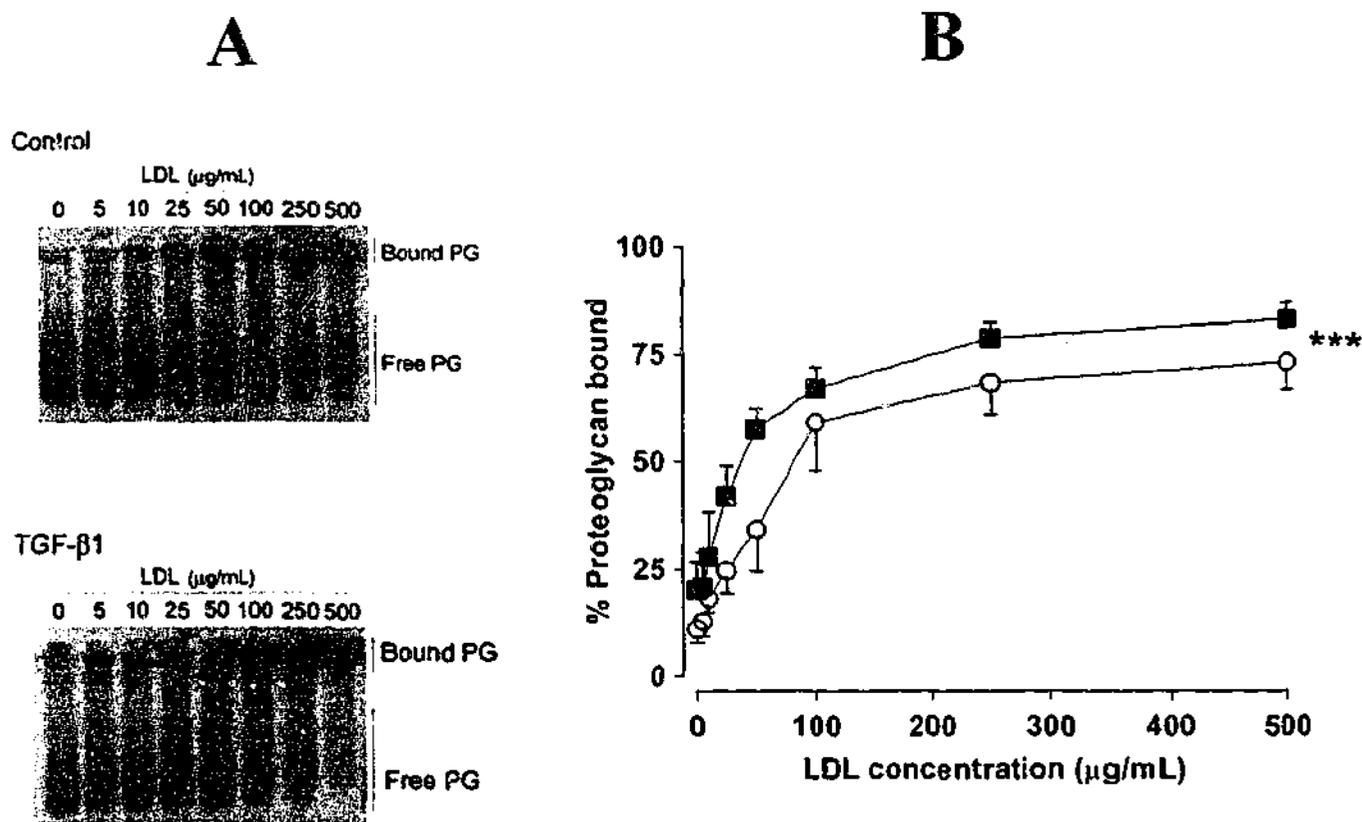
Figure 4-3

Vascular SMCs stimulated with TGF-β1, synthesize proteoglycans that show increased binding to LDL. (A) Human vascular SMCs were metabolically labelled with $[^{35}S]$-met/cys following treatment with TGF-β1 (2 ng/mL). Secreted proteoglycans (PGs) were isolated and equal counts (1500 cpm) were mixed with increasing concentrations of LDL and separated by electrophoresis, on agarose gels. (B) The percent of proteoglycans bound was plotted against the LDL concentration to generate binding curves. Open circles, control cells; closed circles, vascular SMCs treated with TGF-β1 (2 ng/mL). Each data point (n=4) represents mean±SEM of 2 experiments performed in duplicate. Statistical analysis was performed using a paired t-test for the control vs TGF-β1 data sets (**$P<0.01$).

A

Control

LDL (µg/mL)

0 5 10 25 50 100 250 500 Bound PGs / Free PGs

PDGF + Insulin

LDL (µg/mL)

0 5 10 25 50 100 250 500 Bound PGs / Free PGs

B

Y-axis: % Proteoglycan bound

X-axis: LDL concentration (µg/mL)

***
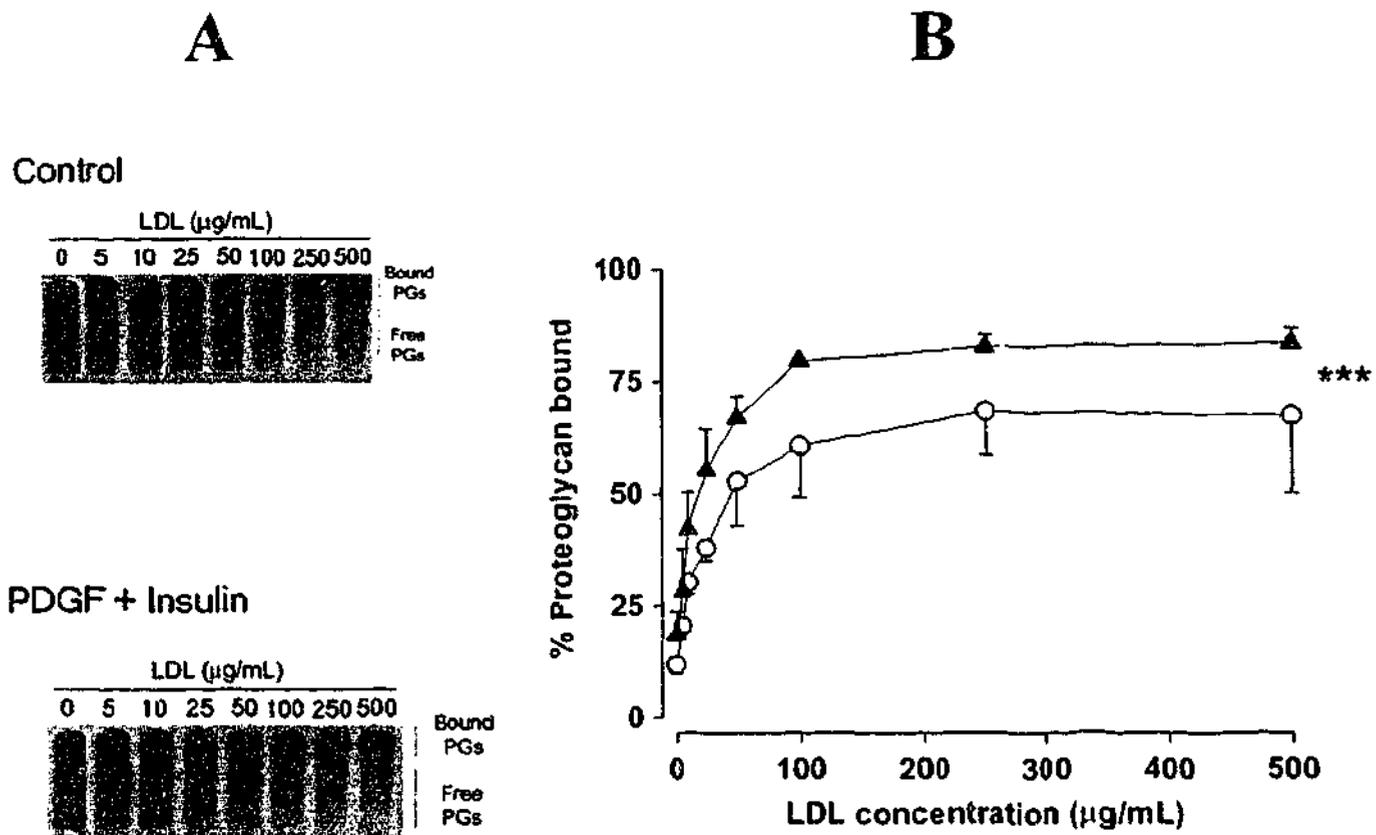
Figure 4-4

Vascular SMCs stimulated with PDGF and insulin, synthesize proteoglycans that show increased binding to LDL. (A) Human vascular SMCs were metabolically labelled with $[^{35}S]$-met/cys in the presence and absence of PDGF (50 ng/mL) plus insulin (1 µmol/L). Secreted proteoglycans were isolated and equal counts (1500 cpm) were mixed with increasing concentrations of LDL and separated by electrophoresis, on agarose gels. (B) The percent of proteoglycans bound was plotted against the LDL concentration to generate binding curves. Open circles, control cells; closed triangles, vascular SMCs treated with PDGF /insulin (50 ng/mL and 1 µmol/L, respectively). Each data point (n=3) represents the mean±SEM of 3 experiments performed in duplicate. Statistical analysis was performed using a paired t-test for the control vs TGF-β1 data sets (**$P<0.01$).

Proteoglycans from vascular SMCs treated with fenofibrate (30 μmol/L) show significantly different ($P<0.01$) binding to LDL compared to vascular proteoglycans synthesized under basal conditions (Figure 4-5A). Treatment of vascular SMCs with fenofibrate increased the half-maximal saturation concentration from the respective control which was 36.8±12.4 μg/mL LDL to 77.7±17.0 μg/mL LDL ($P<0.01$, Figure 4-5A) indicating that the proteoglycans synthesized in the presence of fenofibrate have a reduced affinity for LDL. Vascular SMCs treated with gemfibrozil (100 μmol/L) synthesized proteoglycans that bound to LDL in a manner similar to proteoglycans from untreated cells (Figure 4-5B). The proteoglycans from vascular SMCs treated with fenofibrate (30 μmol/L) in the presence of TGF-β1 showed a different binding curve ($P<0.001$) from proteoglycans produced by SMCs treated with TGF-β1 alone (Figure 4-6A). The proteoglycans synthesized by vascular SMCs in the presence of TGF-β1 and fenofibrate had a half-maximal saturation concentration of 39.1±6.1 μg/mL LDL and this was similar to the half-maximal saturation concentration of proteoglycans from SMCs treated with TGF alone (24.9±4.6 μg/mL LDL, Figure 3-6A). In the presence of TGF-β1 and gemfibrozil (100 μmol/L), the proteoglycans produced by vascular SMCs showed significantly different ($P<0.05$) binding to LDL from proteoglycans synthesized in the presence of TGF-β1 alone (Figure 4-6B). The half-maximal saturation concentration of vascular proteoglycans synthesized in the presence of gemfibrozil and TGF-β1 (23.0±2.7 μg/mL LDL) was similar to the half-maximal saturation concentration of proteoglycans from SMCs treated with TGF-β1 alone (24.9±4.6 μg/mL LDL; Figure 4-6B).

A B
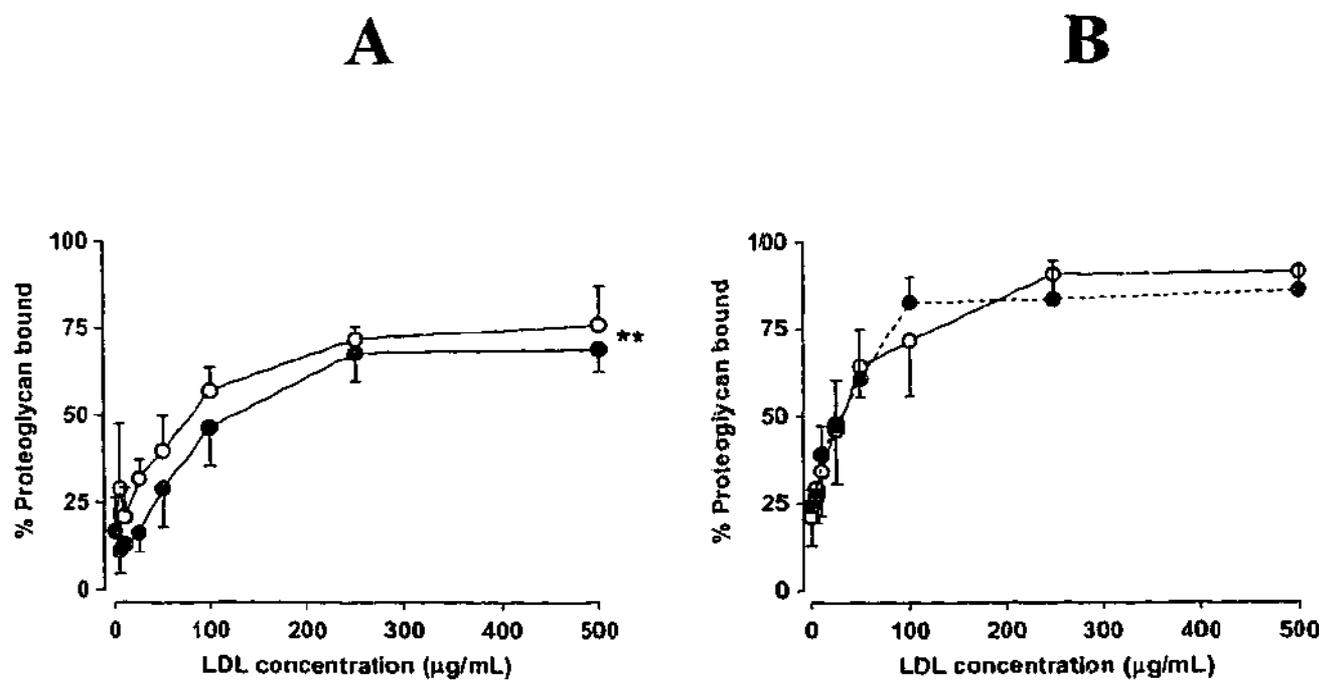
Figure 4-5

Vascular SMCs treated with fenofibrate but not gemfibrozil synthesize proteoglycans that show reduced binding to LDL. Vascular SMC proteoglycans were metabolically labelled with $[^{35}S]$-met/cys in the presence and absence of (A) fenofibrate (30 µmol/L) or (B) gemfibrozil (100 µmol/L). Secreted proteoglycans from vascular SMCs were isolated and equal counts (1500 cpm) were mixed with increasing concentrations of LDL and separated by electrophoresis on agarose gels. The percent of proteoglycans bound was plotted against the LDL concentration to generate binding curves. Open circles, untreated vascular SMCs; closed circles, vascular SMCs treated with (A) fenofibrate (30 µmol/L) or (B) gemfibrozil (100 µmol/L). Each data point (n=2-4) represents the mean±SEM from 1-2 experiments and was analyzed using a paired t-test for the control vs fenofibrate data sets (**$P<0.01$).
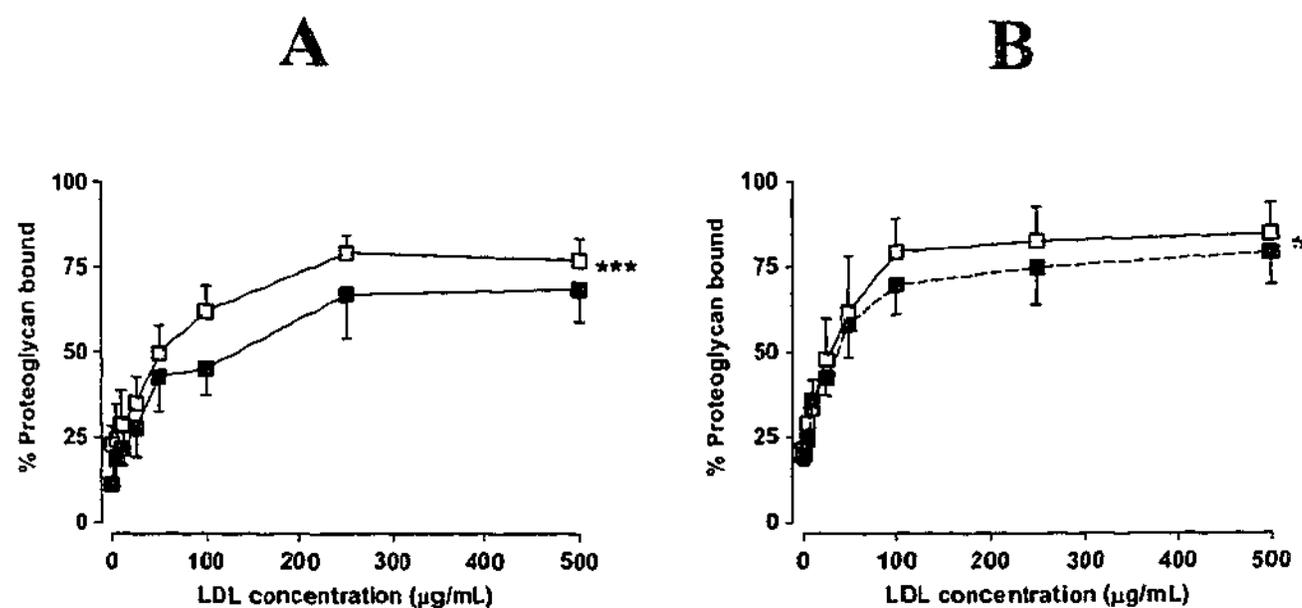
Figure 4-6

Vascular SMCs treated with fenofibrate or gemfibrozil in the presence of TGF-β1 synthesize proteoglycans that show reduced binding to LDL. Vascular SMC proteoglycans were metabolically labelled with $[^{35}S]$-met/cys in the presence and absence of (A) fenofibrate (30 μmol/L) or (B) gemfibrozil (100 μmol/L) with TGF-β1 (2ng/mL). Secreted proteoglycans from vascular SMCs were isolated and equal counts (1500 cpm) were mixed with increasing concentrations of LDL and separated by electrophoresis, on agarose gels. The percent of proteoglycans bound was plotted against the LDL concentration to generate binding curves. Open squares, TGF-β1 treated cells; closed squares, vascular SMCs treated with (A) fenofibrate (30 μmol/L) and TGF-β1 and (B) gemfibrozil (100 μmol/L) with TGF-β1. Each data point (n=2-4) represents the mean±SEM from 1-2 experiments performed in duplicate and was analyzed using a paired t-test for the TGF-β1 vs either TGF-β1+fenofibrate or TGF-β1+gemfibrozil data sets (*$P<0.05$, ***$P<0.001$).

Vascular proteoglycans from SMCs treated with fenofibrate (30 μmol/L) in the presence of PDGF plus insulin (50 ng/mL and 1 μmol/L, respectively) showed significantly different ($P<0.001$) binding to LDL compared to proteoglycans from cells treated with PDGF/insulin alone (Figure 4-7A). Fenofibrate treatment of vascular SMCs in the presence of PDGF/insulin, increased the half-maximal saturation concentration compared to the PDGF/insulin control from 9.5 ± 4.4 μg/mL LDL to 31.1 ± 3.4 μg/mL LDL ($P<0.05$, Figure 4-7A). Vascular proteoglycans from SMCs treated with gemfibrozil in the presence of PDGF/insulin showed significantly different ($P<0.01$) binding to LDL compared to proteoglycans from cells treated with PDGF/insulin alone (Figure 4-7B). Vascular SMCs treated with gemfibrozil increased the half-maximal saturation concentration of LDL compared to vascular proteoglycans synthesized in presence of PDGF/insulin alone from 28.4 ± 0 μg/mL to 9.5 ± 4.4 μg/mL LDL ($P<0.05$; Figure 4-7B). These data show that two compounds which are both fibrates/PPAR-α ligands, modify the fine molecular characteristics of vascular proteoglycans such that their binding to LDL is reduced. The effects are most consistent and pronounced in cells activated by atherogenic growth factors suggesting that fibrates interfere with growth factor signalling which cause atherogenic changes in proteoglycans.
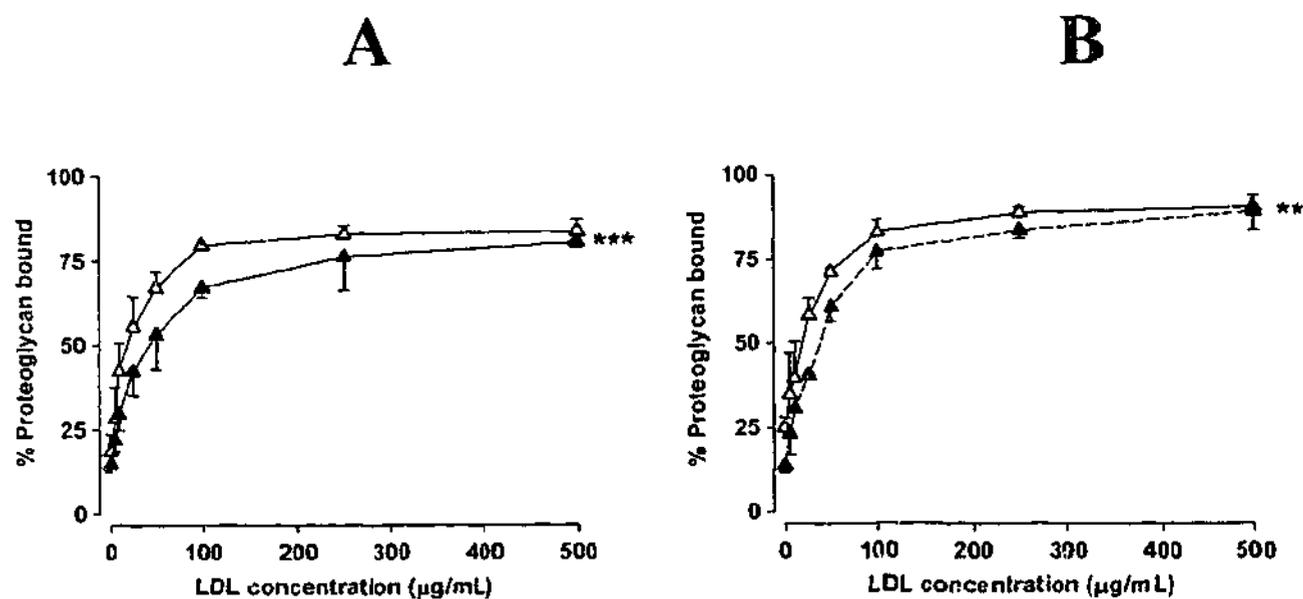
Figure 4-7

Treatment of vascular SMCs with fenofibrate or gemfibrozil in the presence of PDGF/insulin, reduces the binding of proteoglycans to LDL. Vascular SMC proteoglycans were metabolically labelled with $[^{35}S]$-met/cys in the presence and absence of (A) 30 µmol/L fenofibrate or (B) 100 µmol/L gemfibrozil, and each with PDGF plus insulin (50 ng/mL and 1 µmol/L, respectively). Equal counts (1500 cpm) were mixed with increasing concentrations of LDL and separated by electrophoresis through agarose gels. The percent of proteoglycans bound was plotted against the LDL concentration to generate binding curves. Open triangles, PDGF/insulin treated cells; closed squares, vascular SMCs treated with (A) 30 µmol/L fenofibrate and PDGF/insulin and (B) 100 µmol/L gemfibrozil with PDGF/insulin. Data (n=2-3) represents 1-2 experiments performed in duplicate and was statistically analyzed by a paired t-test for the PDGF/insulin vs either PDGF/insulin+fenofibrate or PDGF/insulin+gemfibrozil data sets (**$P<0.01$, ***$P<0.001$).

4.2.3 Interference of proteoglycan:LDL binding – the use of a chondroitin sulfate GAG, heparin and low molecular weight heparins

A recent commentary by Staels (Staels, 2002) relating to a mouse study supporting the "response to retention" hypothesis of atherogenesis (Skalen *et al.*, 2002), included the suggestion that therapies which interfere with the proteoglycan:LDL binding may be useful therapeutics in preventing atherosclerosis. It was suggested that small carbohydrate molecules "GAG mimetics" could disrupt atherogenic LDL binding to proteoglycans. While this proposal is not a specific vascular target, such as the PPAR-α effects on GAG structure and composition presented in Chapter 3, the concept was investigated *in vitro*.

We examined the possibility that a large chondroitin sulfate GAG, heparin, and low molecular weight heparins (enoxaparin, octasaccharide and tetrasaccharide) may interfere with proteoglycan:LDL binding and this was assessed using the gel mobility shift assay. Proteoglycans were obtained from human vascular SMCs maintained under basal conditions that were metabolically labelled with $[^{35}S]$-sulfate. In contrast to earlier experiments with pools of proteoglycans from differently treated vascular SMCs, here an identical pool of proteoglycans is used in each incubation. Hence, the position of the radiolabel in the proteoglycans would not make a difference to the potential displacement of proteoglycan-LDL binding with other GAGs. Proteoglycans were isolated from the culture medium and a fixed amount of proteoglycans (1500 cpm) were mixed with either 25 µg/mL LDL (Figure 4-8) being a low concentration of LDL, 100 µg/mL (Figure 4-9) being a half-maximal saturation concentration of LDL or 500 µg/mL (Figure 4-10) being a maximal saturation concentration of LDL. Increasing amounts (1-100 µg/mL) of chondroitin sulfate, heparin, enoxaparin, octasaccharide and tetrasaccharide were also added to the proteoglycan:LDL mixture. The bound and free proteoglycans were separated on agarose gels, quantitated and expressed as

percentage of proteoglycans bound to LDL (Figures 4-8 to 4-10).

The binding of proteoglycans to LDL (25 μg/mL) was weakly affected by the addition of increasing amounts (1-100 μg/mL) of chondroitin sulfate (Figure 4-8). Increasing amounts of heparin caused an inhibition of proteoglycan-LDL binding in the presence of LDL (25 μg/mL, Figure 4-8). Under these conditions, 30.7% pf proteoglycans bound to 25 μg/mL LDL in the presence of heparin (100 μg/mL) compared to 79.1% of proteoglycans bound to 25 μg/mL LDL without heparin (Figure 4-8). Increasing amounts of enoxaparin, octasaccharide or tetrasaccharide did not affect proteoglycan binding to 25 μg/mL LDL (Figure 4-8). In the presence of 100 μg/mL LDL, 89.9% of proteoglycans were bound (Figure 4-9). Increasing amounts of chondroitin sulfate showed a small effect to interfere with proteoglycan binding with 88.4% of proteoglycans bound to 100 μg/mL LDL in the presence of chondroitin sulfate (100 μg/mL, Figure 4-9). The amount of proteoglycans bound to LDL (100 μg/mL) was r. ..ced to 37.3% in the presence of heparin (100 μg/mL) compared to no heparin (Figure 4-9) as observed under conditions with 25 μg/mL LDL. Enoxaparin and octasaccharide, each at 100 μg/mL, showed interference of proteoglycan binding to 100 μg/mL LDL with 59.7% and 78.0% proteoglycans bound respectively, compared to the control (Figure 4-9). The tetrasaccharide did not disrupt the binding of proteoglycans to LDL (Figure 4-9). In the presence of 500 μg/mL LDL, 95.7% of proteoglycans were bound and this did not change by the addition of increasing amounts of chondroitin sulfate (Figure 4-10). The addition of heparin (100 μg/mL) decreased the amount of proteoglycans bound to 500 μg/mL LDL compared to no heparin (Figure 4-10). The presence of enoxaprin (100 μg/mL) with proteoglycans and 500 μg/mL LDL reduced the amount of proteoglycans bound from 95.7% to 80.4% compared to the control (Figure 4-10). Octasaccharide (100 μg/mL) caused a small

change in the amount of proteoglycans bound (89.0%) compared to the control while tetrasaccharide had no effect on proteoglycan binding to 500 µg/mL LDL (Figure 4-10).

In summary, these data show that heparin has the greatest effect to interfere with the binding of $[^{35}S]$-sulfate labelled proteoglycans to 25 µg/mL, 100 µg/mL and 500 µg/mL LDL. Enoxaparin weakly inhibits the interaction between proteoglycans and LDL while the octasaccharide, tetrasaccharide and the chondroitin sulfate polymer, have virtually no effect on displacing the proteoglycan-LDL complex, using this assay (Figure 4-8).

25 µg/mL LDL
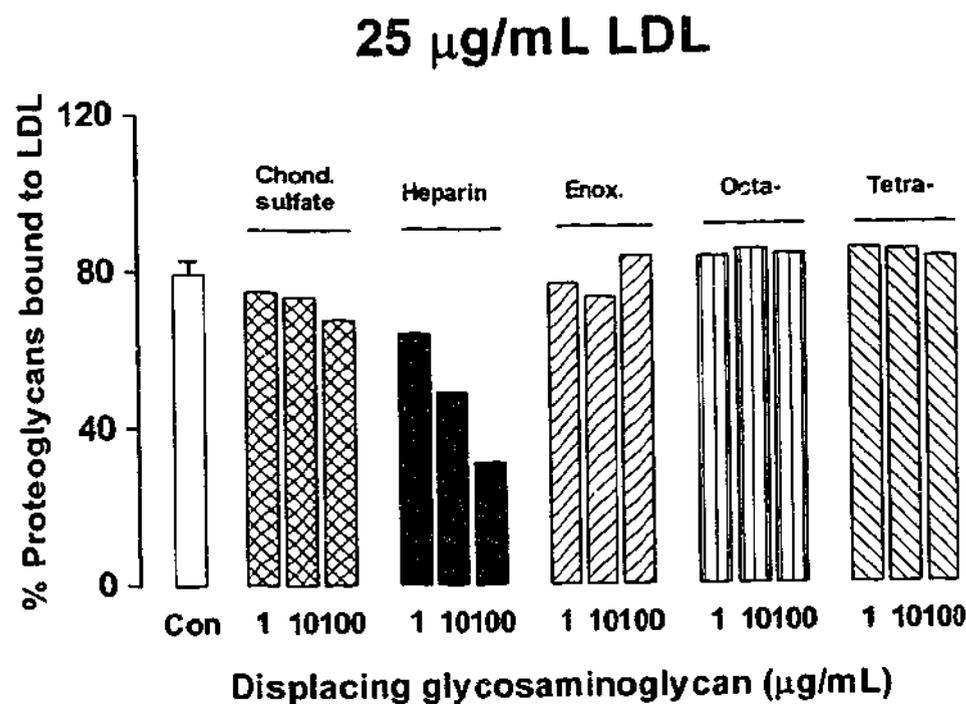
Figure 4-8

Heparin disrupts proteoglycan:LDL binding at a low (25 µg/mL) concentration of LDL.

Chondroitin (Chond.) sulfate, heparin, enoxaparin (Enox.), octasaccharide (Octa-) and tetrasaccharide (tetra-) each at 1, 10 and 100 µg/mL were incubated with 25 µg/mL of normal human LDL-cholesterol in the presence of a fixed amount (1500 cpm) of [^{35}S]-sulfate labelled proteoglycans from human vascular SMCs. Bound and free proteoglycans were separated on a flatbed, agarose (0.8%) gel at 4°C for 2 hours. Gels were air-dried and images were captured using a phosphorimager. Bound and free proteoglycans were quantitated for each condition to express the percentage of proteoglycans bound to LDL. The control (Con) was the mean±SEM of 3 separate lanes containing LDL and proteoglycans only.

100 µg/mL LDL
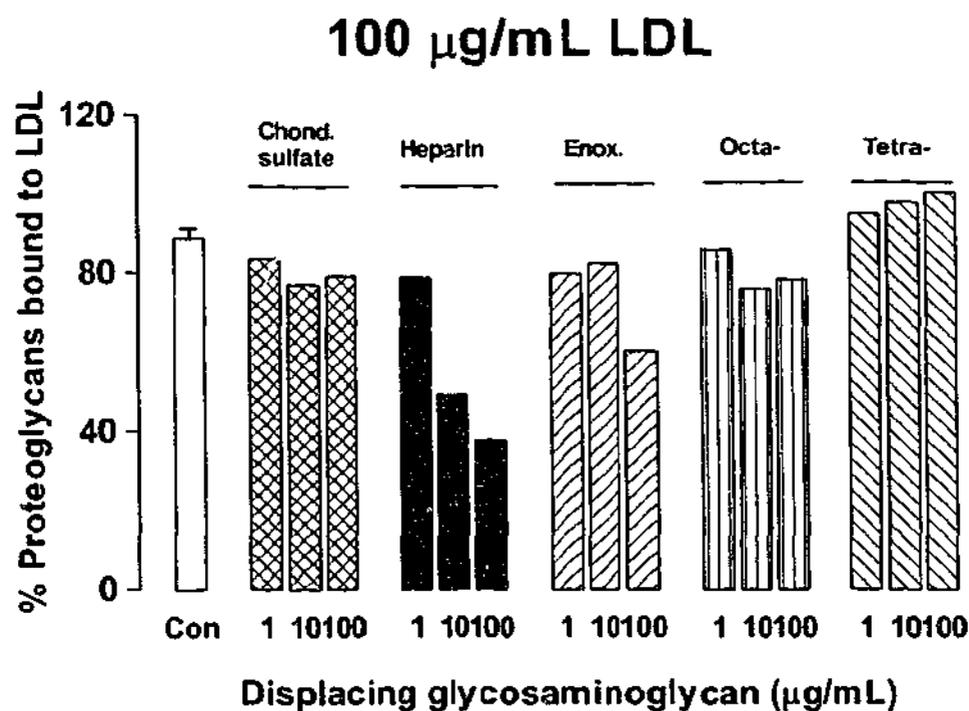
Figure 4-9

Heparin disrupts proteoglycan:LDL at a sub-maximal (100 µg/mL) concentration of LDL. Chondroitin (Chond.) sulfate, heparin, enoxaparin (Enox.), octasaccharide (Octa-) and tetrasaccharide (tetra-) each at 1, 10 and 100 µg/mL were incubated with 100 µg/mL of normal human LDL-cholesterol in the presence of a fixed amount (1500 cpm) of [^{35}S]-sulfate labelled proteoglycans from human vascular SMCs. The gel mobility shift assay was performed and the data obtained as described for Figure 4-8. Data are from one experiment. The control (Con) was the mean±SEM of 3 separate lanes containing LDL and proteoglycans only.

500 μg/mL LDL
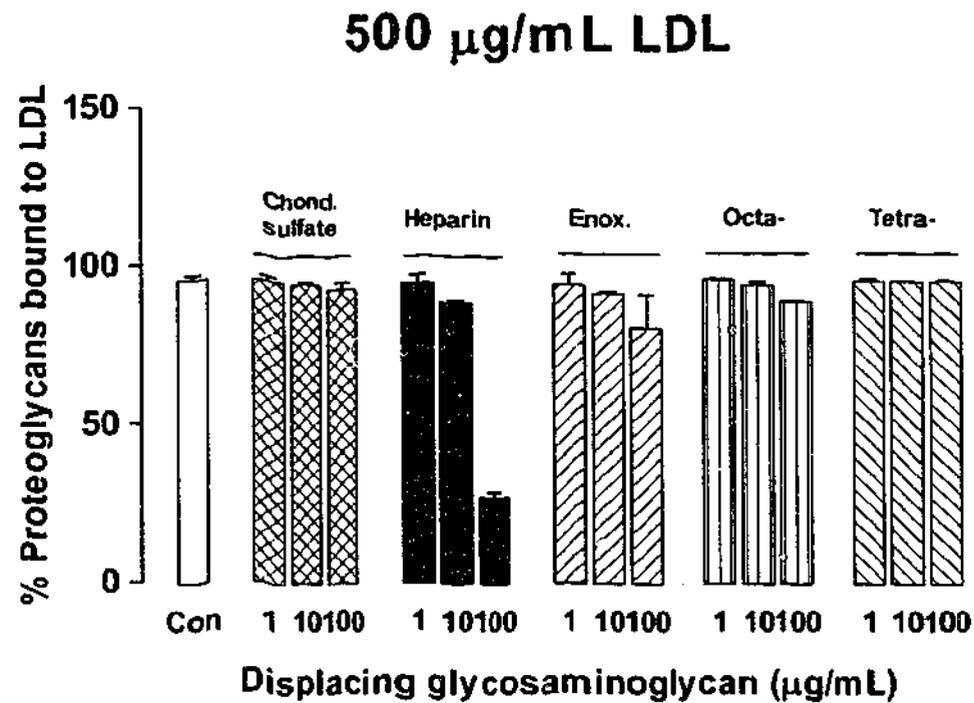
Figure 4-10

Heparin disrupts proteoglycan:LDL at a maximal (500 μg/mL) concentration of LDL.
Chondroitin (Chond.) sulfate, heparin, enoxaparin (Enox.), octasaccharide (Octa-) and tetrasaccharide (tetra-) each at 1, 10 and 100 μg/mL were incubated with 500 μg/mL of normal human LDL-cholesterol in the presence of a fixed amount (1500 cpm) of [^{35}S]-sulfate labelled proteoglycans from human vascular SMCs. The gel mobility shift assay was performed and the data obtained as described for Figure 4-8. Data are mean±SD of 2 experiments. The control (Con) was the mean±SEM of 3 separate lanes containing LDL and proteoglycans only.

4.3 Summary of results for Chapter 4

In this chapter the work presented is directly related to the "response to retention" hypothesis of atherogenesis by assessing proteoglycan-LDL binding using the gel mobility shift assay. These studies have shown that:

- The GMSA is saturable in terms of both the concentration of LDL and the amount of proteoglycans;

- Treatment of vascular SMCs with TGF-β1 or the combination of PDGF with insulin results in the synthesis of proteoglycans that show enhanced binding to LDL;

- Under basal conditions, the treatment of vascular SMCs with fenofibrate but not gemfibrozil results in the production of proteoglycans that show reduced binding to LDL;

- In the presence of TGF-β1 or PDGF/insulin, both fenofibrate and gemfibrozil treated SMCs secreted proteoglycans that showed reduced binding to LDL;

- Heparin is capable of displacing proteoglycan-LDL binding *in vitro* in a concentration-related manner.

4.4 Discussion of results for Chapter 4

The preparations of proteoglycans from human vascular SMCs contain predominantly biglycan and decorin (see Chapter 3, Figure 3-20 and Table 3-2), which have been previously shown to co-localize with LDL in human atherosclerotic plaques (O'Brien *et al.*, 1998). Hence, the study of these proteoglycans and their binding to LDL by the GMSA is a practical *in vitro* method for showing possible interactions that may occur in the vessel wall. The data in this chapter shows that the gel mobility shift assay is an appropriate method for analyzing the changes in binding of proteoglycans to LDL. The GMSA has been optimized for the purpose of demonstrating that changes in GAG length, composition or charge will result in an alteration in binding to LDL. The main considerations for the GMSA are the choice of radiolabel and the demonstration of saturability.

The data in this chapter has shown that human vascular SMCs stimulated with TGF-β1 synthesize proteoglycans that show increased binding to LDL compared to proteoglycans from cells maintained under basal conditions. These data confirm earlier findings from our laboratory on enhanced LDL binding with proteoglycans from monkey vascular SMCs treated with TGF-β1 (Little *et al.*, 2002). Additionally the data presented here expands the knowledge of the effect of PDGF to increase GAG length (Schonherr *et al.*, 1993; Schonherr *et al.*, 1991) by showing that vascular SMCs treated with both PDGF and insulin, synthesize proteoglycans that show increased binding to LDL compared to untreated cells.

The treatment of vascular SMCs with fenofibrate but not gemfibrozil under basal conditions resulted in the synthesis of proteoglycans that showed reduced binding to LDL. The binding of LDL to proteoglycans synthesized by vascular SMCs treated with TGF-β1 in the presence of fenofibrate was significantly different from the binding of proteoglycans synthesized in the presence of TGF-β1 alone. Similarly, proteoglycans produced by human

vascular SMCs treated with gemfibrozil in the presence of TGF-β1 bound less to LDL than proteoglycans from cells treated with TGF-β1 alone. Reduced binding of LDL to vascular proteoglycans synthesized in the presence of fenofibrate or gemfibrozil with TGF-β1 was also observed when cells were treated with the alternative combination of PDGF/insulin. These data indicate that fibrates have effects to reduce proteoglycan-LDL binding *in vitro*. If these beneficial changes were reproduced *in vivo*, fenofibrate (and other fibrates) may reduce the development of atherogenesis.

The "response to retention" hypothesis was also investigated on the basis of a recent proposal which indicated that "GAG mimetics" may compete with vascular proteoglycans for LDL as a means of reducing LDL retention in the vessel wall (Staels, 2002). We observed that there was no significant effect of chondroitin sulfate (CS) to interfere with proteoglycan binding at all the concentrations of LDL studied. The CS product used contained predominantly (70%) chondroitin sulfate A while the remainder was chondroitin sulfate C. Chondroitin sulfate A is sulfated at the C6 position while chondroitin sulfate C is sulfated at the C4 position. The position of the sulfate group in the CS used in this assay may explain why there was little inhibition of proteoglycan-LDL binding. Glycosaminoglycans with 4-sulfation have been shown to bind to LDL with higher affinity than 6-sulfated GAGs (Gigli *et al.*, 1993). The GAGs in our preparations of proteoglycans contain predominantly 4-sulfated disaccharides as detected by FACE (see Chapter 3, Figure 3-23), hence the addition of exogenous chondroitin sulfate A does not have the physical ability to disrupt the proteoglycan-LDL complex. Another possible explanation for the absence of interference of proteoglycan-LDL binding with CS may be due to the structural stability of the intact proteoglycan molecule compared to the free CS GAG. Little *et al.* (Little *et al.*, 2002) have shown that there is no binding of the core protein to LDL however the intact proteoglycan has

a half-maximal saturation concentration of 81 μg/mL LDL which is at least 1 order of magnitude greater than for secreted free chains at 1350 μg/mL LDL. This study suggested that the core protein may give some structural stability to proteoglycan-LDL complex thereby making interference with a free CS GAG less stable, which may explain why we did not observe displacement of proteoglycan-LDL binding with the addition of CS GAG. Heparin caused the greatest inhibition of proteoglycan-LDL binding which supports previous work showing that the binding of heparin>CS>DS>HS to LDL or other positively charged proteins (Fager et al., 1995) and this is due to the heavily sulfated nature and high iduronic acid content of heparin (See Chapter 1, Figure 1-14B). The smaller heparin-like molecules did not show the same potency as heparin suggesting that they may have different sulfation patterns, contain less iduronic acid or simply cause less displacement of LDL because they are smaller than the core protein associated vascular GAGs.

Chapter 5: *Hyaluronan, glucosamine and glucose metabolism – the effect of PPAR-α and -γ ligands on vascular smooth muscle cells*

5.1 Introduction to results for Chapter 5

The work presented to this point has shown that the treatment of vascular SMCs with fenofibrate in the presence of atherogenic growth factors results in a reduction in $[^{35}S]$-sulfate incorporation into secreted proteoglycans with a concomitant reduction in GAG length. To further assess glycosaminoglycan synthesis, fenofibrate treated vascular SMCs were metabolically labelled with $[^{3}H]$-glucosamine. Treatment of human vascular SMCs with fenofibrate (50 μmol/L) resulted in an unexpected increase in the incorporation of $[^{3}H]$-hexosamine into total GAGs (Chapter 3, Section 3.2.6). Under some conditions the pattern of $[^{35}S]$-sulfate incorporation may be different from the $[^{3}H]$-glucosamine incorporation data. For example, the treatment of fibroblasts or vascular SMCs with sodium chlorate, inhibits $[^{35}S]$-sulfate incorporation into GAGs but has no effect on $[^{3}H]$-glucosamine incorporation into GAGs and this is due to sodium chlorate specifically inhibiting 4-sulfation (Ballinger et al., 2004; Phamantu et al., 1999). Fenofibrate treatment of SMCs showed no effect on the sulfation pattern of GAGs (Chapter 3, Section 3.2.10). The observed increase in $[^{3}H]$-hexosamine incorporation into total GAGs may possibly arise from an increase in GAG chain length however, this possibility is excluded by the SDS-PAGE and size exclusion chromatography data (Chapter 3, Sections 3.2.7-3.2.9) which shows that vascular SMCs treated with fenofibrate produced proteoglycans with reduced GAG length compared to untreated cells. Secondly, an increase in $[^{3}H]$-hexosamine incorporation into total GAGs may occur if the treatment of vascular SMCs with fenofibrate, increased the amount of hyaluronan synthesized. A third possibility for the increased incorporation of $[^{3}H]$-hexosamine into total GAGs would be that the treatment of vascular SMCs with fenofibrate has altered the specific activity of the radiolabelled glucosamine precursors used for GAG synthesis. To determine how fenofibrate treatment increases the incorporation of $[^{3}H]$-hexosamine into total GAG, we

have investigated the latter 2 possibilities: hyaluronan production and the specific activity of radiolabelled glucosamine.

Most glycosaminoglycans (GAGs) which are covalently linked to a core protein or non-protein associated GAG of hyaluronan, are polysaccharide chains composed of linking disaccharide units. Each disaccharide is composed of a hexosamine (glucosamine or galactosamine) and an uronic acid (glucuronic or iduronic acid). The hexosamines which form GAG chains are derived from the hexosamine pathway. The hexosamine pathway involves the phosphorylation of glucose, fructose or mannose and conversion to phosphorylated GlcNAc (Yanagishita *et al.*, 1989). Subsequently, GlcNAc is converted to UDP-GlcNAc and then UDP-N-acetylgalactosamine (UDP-GalNAc). Exogenous glucosamine, including [^{3}H]-glucosamine, is directly phosphorylated and *N*-acetylated to form [^{3}H]-UDP-GlcNAc and [^{3}H]-UDP-GalNAc which are incorporated into glycosaminoglycans of proteoglycans and hyaluronan.

The specific activity of a tracer is the radioactivity of the label per unit mass of the total chemical entity. Metabolic labelling of cells with radiolabelled glucosamine has shown that there is an inverse relationship between the specific activity of glucosamine and the concentration of glucose or glucosamine in the culture medium (Kim & Conrad, 1976; Yanagishita & Hascall, 1985). For example, studies in chick embryo chondrocytes showed that a depletion in the glucose concentration in the culture medium, progressively increased the specific activity of [^{14}C]-glucosamine into GAGs on proteoglycans (Kim & Conrad, 1976). Other cellular conditions where the specific activity of glucosamine is increased, with an associated reduction in [^{35}S]-sulfate incorporation into proteoglycans, is when the cellular pH is elevated by monensin treatment of rat ovarian granulosa cells (Yanagishita & Hascall, 1985).

The treatment of chick embryo vertebral cartilage with a high concentration (25 mmol/L) of glucosamine, followed by metabolic labelling with [^{14}C]-glucosamine showed that there was a reduction in the incorporation of [^{14}C]-glucosamine into GAGs on proteoglycans, indicating a reduction in the specific activity of glucosamine however, the reduction in [^{14}C]-glucosamine incorporation into proteoglycans may also be a reflection of a reduction in total protein synthesis (Kim & Conrad, 1974). These data are supported by a study in vascular SMCs which showed that treatment with glucosamine (2-32 mmol/L) results in a concentration-related reduction in [^{35}S]-sulfate incorporation into proteoglycans which is associated with a decrease in GAG synthesis and length (Tannock *et al.*, 2002a).

Changes in the specific activity of radiolabelled glucosamine have not been measured following metabolic labelling of human vascular smooth muscle cells, nor has the relatively new technique of fluorophore assisted carbohydrate electrophoresis (FACE) (Calabro *et al.*, 2000a; Calabro *et al.*, 2000b) been used to assess this phenomenon. To assess if the effects of fibrates/PPAR-α to increase the incorporation of [^{3}H]-hexosamine into GAGs may be general for cell activation by PPARs, we included a PPAR-γ ligand (troglitazone) in the experiments. The incorporation of [^{35}S]-sulfate and [^{3}H]-glucosamine into disaccharides of CS/DS GAGs synthesized by human vascular SMCs following treatment with the PPAR ligands, was assessed by FACE. Additionally, the effect of fenofibrate/troglitazone treatment on vascular SMC hyaluronan synthesis and mass is also presented.

5.2   Results of Chapter 5

5.2.1   Quantitation and electrophoretic migration by SDS-PAGE of proteoglycans synthesized by fenofibrate and troglitazone treated vascular SMCs

To confirm the data presented in Chapter 3, Section 3.2.6, that metabolic labelling of

human vascular SMCs following treatment with fenofibrate increases the incorporation of [^{3}H]-glucosamine into GAGs but decreases the incorporation of [^{35}S]-sulfate, these experiments were repeated and expanded to include troglitazone, a PPAR-γ ligand. Human vascular SMCs treated with either fenofibrate (30 µmol/L) or troglitazone (10 µmol/L), were metabolically labelled with [^{35}S]-sulfate (50 µCi/mL) and separately with [^{3}H]-glucosamine (10 µCi/mL) for the quantitation of proteoglycans. [^{14}C]-Glucosamine (50 µCi/mL) was used in preference to [^{3}H]-glucosamine to assess the electrophoretic mobility of proteoglycans because the former label gave phosphor-luminescent images in a more practical time. After 24 h the culture medium was assayed for total incorporation of isotopes into secreted glycosaminoglycans by the CPC precipitation assay (Chapter 2, Section 2.5) and proteoglycans were prepared for separation by SDS-PAGE (Chapter 2, Section 2.7).

We have confirmed the results of Nigro *et al.* (Nigro *et al.*, 2002) and Tannock *et al.* (Tannock *et al.*, 2004) that under low glucose (5.5 mmol/L) conditions, there was no significant (n.s) change in sulfate incorporation into proteoglycans following treatment of vascular SMCs with fenofibrate (30 µmol/L) while there was a 22.3% (not significant) decrease in sulfate incorporation into proteoglycans following treatment of vascular SMCs with troglitazone (10 µmol/L; Figure 5-1A), respectively. The effect of fenofibrate and troglitazone to decrease [^{35}S]-sulfate incorporation into proteoglycans under low glucose conditions was similar under high glucose conditions with 14.9% (not significant) and 31.3% (*P*<0.05) reduction in [^{35}S]-sulfate incorporation, respectively compared to the high glucose control (Figure 5-1A). The reductions in sulfate incorporation observed were associated with an increase in the electrophoretic mobility of proteoglycans by SDS-PAGE, which was more apparent following treatment of vascular SMCs with troglitazone compared to fenofibrate (Figure 5-1B). It is interesting to compare the effects of PPAR-α and PPAR-γ ligands at near

maximally tolerated concentrations. Treatment of vascular SMCs with troglitazone has a greater effect at 10 μmol/L than fenofibrate at 30 μmol/L, on reducing sulfate incorporation into proteoglycans and reducing GAG length (Figure 5-1A).

Vascular SMCs treated with either fenofibrate (30 μmol/L) or troglitazone (10 μmol/L) and metabolically labelled with [^{3}H]-glucosamine showed an increase in incorporation of [^{3}H]-hexosamine into GAGs by 10.4% (n.s) and 29.8% ($P<0.01$), respectively compared to the low glucose control (Figure 5-2A). The effect of the PPAR ligands to stimulate [^{3}H]-glucosamine incorporation, under low glucose conditions, was not observed in the presence of high (25 mmol/L) glucose medium (Figure 5-2A). The increase in [^{3}H]-hexosamine incorporation into GAGs following the treatment of vascular SMCs with fenofibrate or troglitazone under low glucose conditions might suggest that there is an increase in the number of proteoglycan core proteins and/or a potential increase in GAG length. In earlier studies it was shown that the treatment of human vascular SMCs with fenofibrate increased proteoglycan core protein synthesis by ≈16% (Chapter 3, Section 3.2.3) however, under similar conditions, these findings did not account for the 95.6% increase in [^{3}H]-glucosamine incorporation into GAGs (Chapter 3, Section 3.2.6). Therefore, we chose to assess whether the increase in [^{3}H]-glucosamine incorporation with the PPAR ligands affected the length of the GAGs by metabolic labelling with [^{14}C]-glucosamine and assessing the electrophoretic migration by SDS-PAGE. As expected and observed with [^{35}S]-sulfate labelled proteoglycans, vascular SMCs treated with troglitazone and to a lesser extent with fenofibrate resulted in the synthesis of [^{14}C]-glucosamine labelled proteoglycans with an apparent reduction in GAG length (Figure 5-2B). These data suggest that the increase in [^{3}H]-glucosamine incorporation following treatment with fenofibrate or troglitazone is not due to an increase in GAG length however, there may be an increase in hyaluronan synthesis or a

change in the specific activity of glucosamine under low glucose conditions and these two possibilities were investigated.

It was apparent that the glucose concentration in the culture medium of vascular SMCs modified the incorporation of [^{35}S]-sulfate and [^{3}H]-glucosamine into secreted proteoglycans. Vascular SMCs incubated with high (25 mmol/L) glucose medium, showed a 21.6% (n.s) increase in [^{35}S]-sulfate incorporation into proteoglycans compared to cells incubated in the presence of low (5.5 mmol/L) glucose medium (Figure 5-1A). The comparison of vascular SMCs metabolically labelled in low glucose medium to those labelled under high glucose conditions showed that the incorporation of [^{3}H]-hexosamine into GAGs was reduced by 34.2% ($P<0.001$) under high glucose conditions compared to the low glucose control (Figure 5-2A). The sulfate incorporation data suggests that high glucose may stimulate proteoglycan synthesis. However, under these conditions the glucosamine pool may be diluted by glucose metabolic products hence, the specific activity of [^{3}H]-glucosamine is reduced and this is reflected as decreased [^{3}H]-hexosamine incorporation into GAGs.
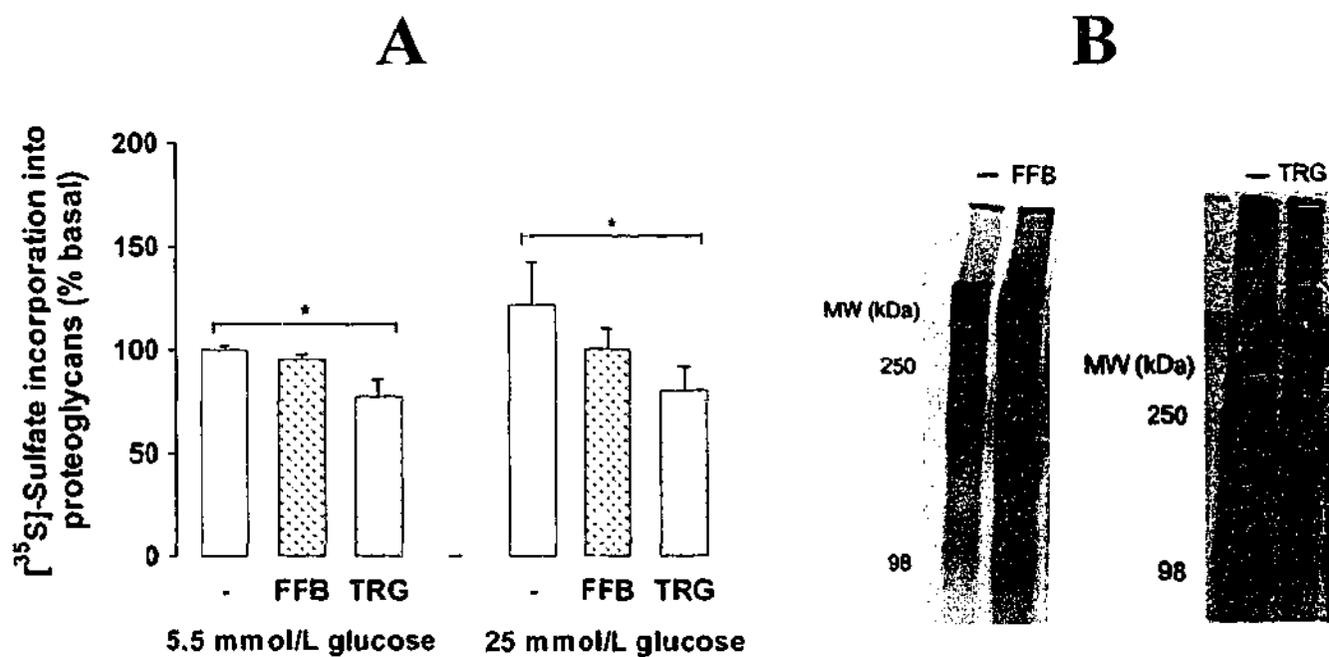
Figure 5-1

Treatment of vascular SMCs with PPAR ligands reduces [^{35}S]-sulfate incorporation into proteoglycans and increases their electrophoretic mobility by SDS-PAGE. (A) Human vascular SMCs were treated with 0.1% DMSO (-), fenofibrate (30 µmol/L; FFB) or troglitazone (10 µmol/L; TRG) in culture medium containing 5.5 mmol/L glucose or 25 mmol/L glucose and were metabolically labelled with [^{35}S]-sulfate. Secreted vascular proteoglycans were quantitated by the CPC precipitation assay. Data is expressed as the mean±SEM of 2 experiments, normalized to the low glucose control (167.6±10.1 cpm [^{35}S]-sulfate/10^3 cells). Data was analyzed using a Student's t-test and a 1-way ANOVA (*$P<0.05$). (B) Vascular proteoglycans, from SMCs treated with 0.1% DMSO (-), fenofibrate (50 µmol/L) or troglitazone (10 µmol/L) and metabolically labelled with [^{35}S]-sulfate under low (5.5 mmol/L) glucose conditions, were separated by SDS-PAGE. Gels represent results from 2-3 experiments with similar results.
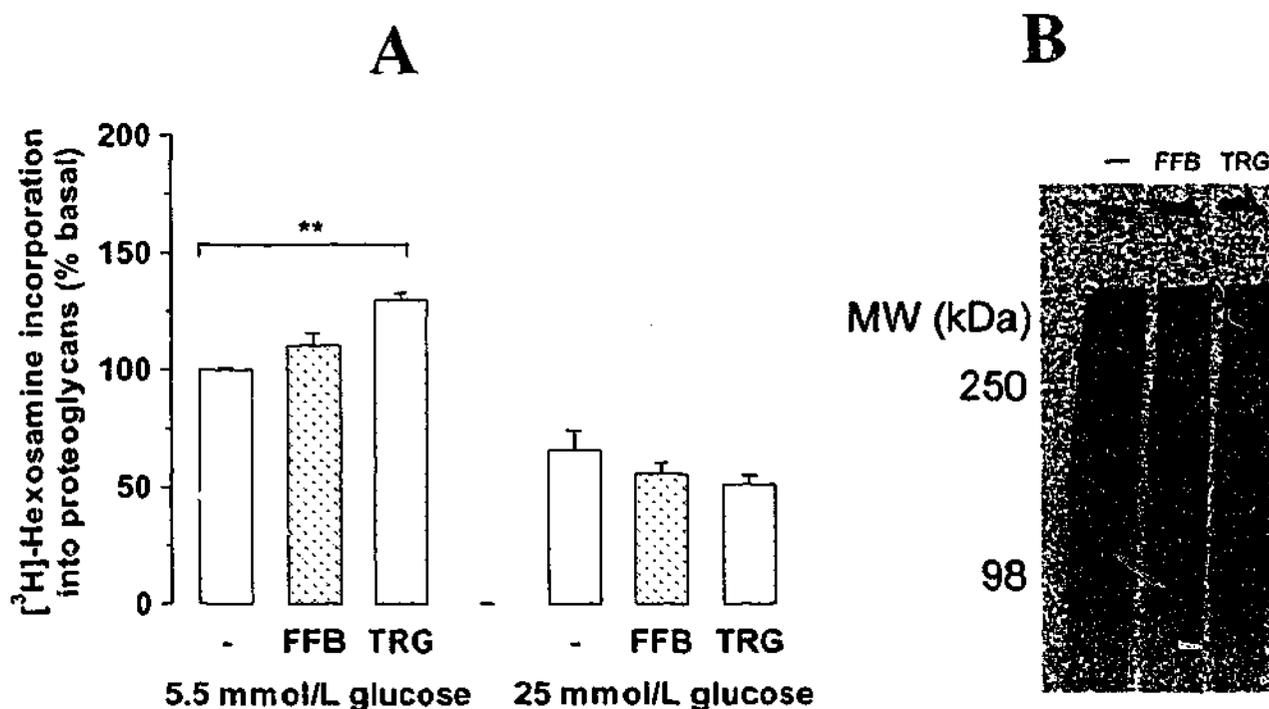
Figure 5-2

Treatment of vascular SMCs with PPAR ligands increases [³H]-glucosamine incorporation into proteoglycans while maintaining an increase in the electrophoretic mobility by SDS-PAGE. (A) Human vascular SMCs were treated with 0.1% DMSO (-), fenofibrate (30 µmol/L; FFB) or troglitazone (TRG; 10 µmol/L) in culture medium containing 5.5 mmol/L or 25 mmol/L glucose and were metabolically labelled with [³H]-glucosamine. Secreted vascular proteoglycans were quantitated using the CPC precipitation assay. Data is expressed as the mean±SEM of 2 experiments normalized to the low glucose control (263.4±7.4 cpm [³H]-hexosamine/10^3 cells). Data was analyzed using a 1-way ANOVA (** $P<0.01$). (B) Vascular proteoglycans, from SMCs treated with 0.1% DMSO (-), fenofibrate (30 µmol/L) or troglitazone (10 µmol/L) and metabolically labelled with [¹⁴C]-glucosamine under low (5.5 mmol/L) glucose conditions, were separated by SDS-PAGE. Gel represents results from one experiment.

5.2.2 Assessment of hyaluronan synthesis following treatment of vascular SMCs with PPAR ligands

The CPC assay precipitates total GAG including proteoglycans and hyaluronan on Whatman 3MM paper with 1% CPC and 50 mmol/L NaCl hence, the anomalous increase in [^{3}H]-hexosamine incorporation into GAGs following treatment of vascular SMCs with fenofibrate, could possibly arise from a stimulation of hyaluronan synthesis. We used analytical DEAE-Sephacel and the hyaluronan-binding region colorimetric mass assay to assess whether the increase in glucosamine incorporation into CPC precipitable material following the treatment of vascular SMCs with fenofibrate was due to a stimulation of hyaluronan synthesis.

For the analysis of hyaluronan, vascular SMCs derived from the thoracic aorta of the monkey (*Macaca nemestrina*), kindly provided by Dr. Elaine Raines (University of Washington, Seattle, WA, USA), were used. Vascular SMCs were metabolically labelled with both [^{3}H]-glucosamine (15 μCi/mL) and [^{35}S]-sulfate (100 μCi/mL) following treatment with the PPAR-α ligand, fenofibrate. The [^{3}H]-glucosamine label is incorporated into acidic glycoproteins (labelled peak 1; see Figure 5-3 and 5-4), hyaluronan (labelled peak 2) and proteoglycans (labelled peak 3), while the [^{35}S]-sulfate is only incorporated into proteoglycans. A linear gradient of NaCl (0-0.7 mol/L) was used to separate the glycoproteins, hyaluronan and proteoglycans, providing information about the ionic charge of the metabolically labelled molecules (Chapter 2, Section 2.12). The NaCl elution ranges for hyaluronan and proteoglycans from each treatment group, along with the hyaluronan to proteoglycan ratios are summarized in Table 5-1. Hyaluronan synthesized by vascular SMCs maintained under basal conditions, eluted at a NaCl concentration range of 0.10-0.20 mol/L (Figure 5-3A and Table 5-1). The treatment of vascular SMCs with fenofibrate did not alter

the ionic charge of hyaluronan compared to untreated cells (Figure 5-3B and Table 5-1). Treatment of vascular SMCs with TGF-β1 increased the ionic strength for elution of the hyaluronan peak compared to untreated cells, from 0.10-0.20 mol/L NaCl to 0.10-0.28 mol/L NaCl (Figure 5-4A and Table 5-1). Hyaluronan from vascular SMCs treated with fenofibrate in the presence of TGF-β1 had a modest reduction in ionic strength compared to cells treated with TGF-β1 alone, with an elution range of 0.10-0.24 mol/L NaCl (Figure 5-4B and Table 5-1).

To expand the analysis of the data, we calculated the HA:proteoglycan ratio by using the area under the hyaluronan peak (peak 2) and the area under the proteoglycan peak (peak 3). Under basal conditions, the ratio of hyaluronan to proteoglycans was 0.28±0.06 and this was reduced to 0.12±0.05 following the treatment of vascular SMCs with fenofibrate (Table 5-1). There was a 3-fold increase in the HA:PG ratio following stimulation of vascular SMCs with TGF-β1 compared to untreated cells (Table 5-1). Vascular SMCs treated with fenofibrate in the presence of TGF-β1 showed a reduction in the ratio of HA to proteoglycans compared to cells treated with TGF-β1 alone from 0.86±0.46 to 0.19±0.04 (Table 5-1). These data therefore suggest that the increase in [^{3}H]-glucosamine incorporation into CPC precipitable material following treatment of SMCs with fenofibrate, is not due to an increase in hyaluronan synthesis. Thus, fenofibrate treatment inhibits rather than stimulates hyaluronan biosynthesis.

Untreated SMCs

A

Fenofibrate treated SMCs

B
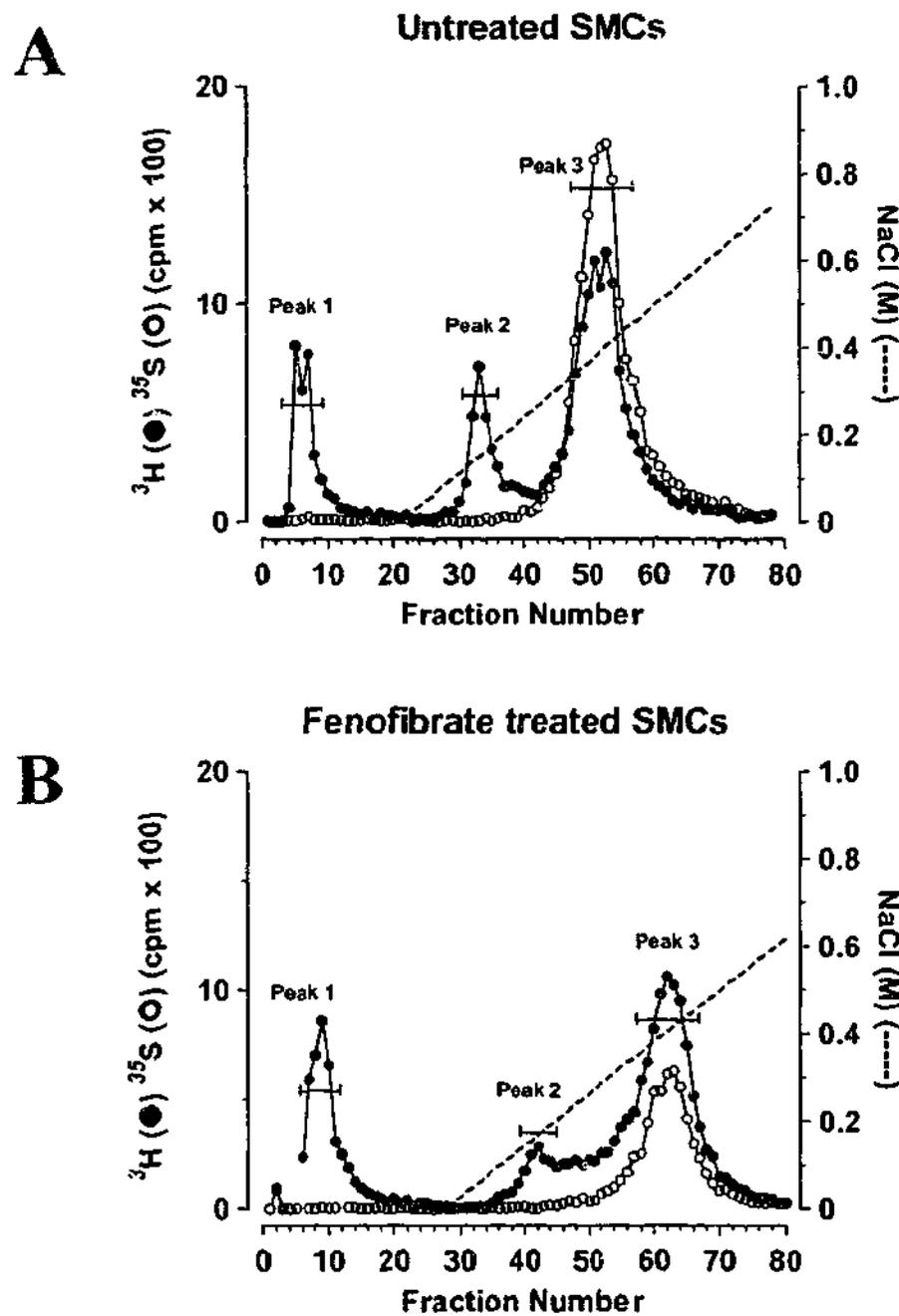
Figure 5-3

Fenofibrate treatment of monkey SMCs reduces hyaluronan synthesis. Macromolecules in the culture medium from monkey vascular SMCs (A) maintained under basal conditions or (B) treated with fenofibrate (30 μmol/L) were separated on an analytical DEAE column. Acidic glycoproteins (peak 1) and hyaluronan (peak 2) are metabolically labelled with [^{3}H]-glucosamine (solid circles) and proteoglycans (peak 3) are metabolically labelled with both [^{3}H]-galactosamine and [^{35}S]-sulfate (open circles). These analytical DEAE profiles are a representative of 2 experiments.
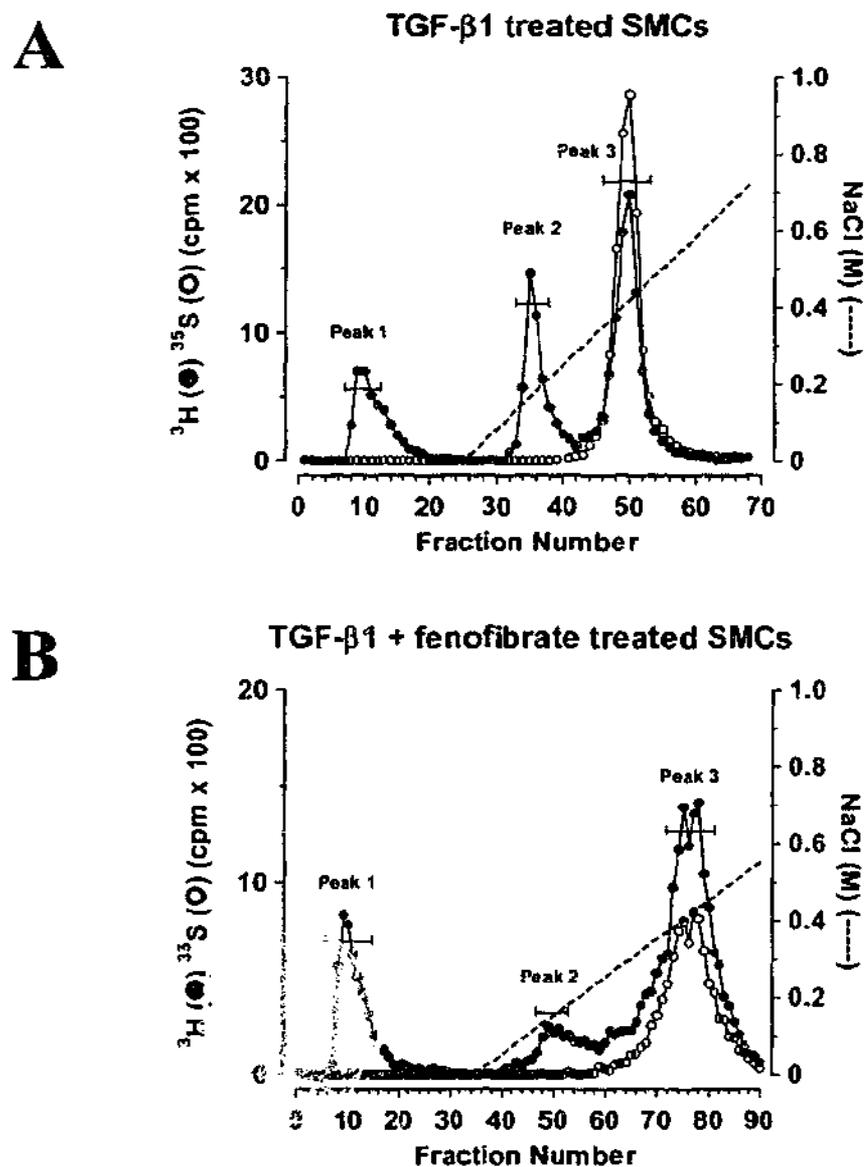
Figure 5-4

Fenofibrate treatment of monkey SMCs in the presence of TGF-β1 reduces hyaluronan synthesis. Macromolecules in the culture medium from monkey vascular SMCs treated with (A) TGF-β1 (2 ng/mL) or (B) fenofibrate (30 μmol/L) in the presence of TGF-β1 (2 ng/mL), were separated on an analytical DEAE column. Acidic glycoproteins (peak 1) and hyaluronan (peak 2) are metabolically labelled with [³H]-glucosamine (solid circles) and proteoglycans (peak 3) are metabolically labelled with [³H]-galactosamine (solid circles) and [³⁵S]-sulfate (open circles). These analytical DEAE profiles are a representative of 2 experiments.

Treatment	NaCl elution range (mol/L)		
	Hyaluronan (peak 2)	Proteoglycans (peak 3)	Ratio of HA:PGs
Basal	0.10 - 0.20	0.26 – 0.57	0.28 ±0.06
Fenofibrate (30 μmol/L)	0.10 – 0.20	0.29 – 0.56	0.12 ± 0.05
TGF-β1	0.10 – 0.28	0.32 – 0.55	0.86 ± 0.46
TGF-β1 + Fenofibrate (30 μmol/L)	0.10 – 0.24	0.30 – 0.55	0.19 ± 0.04

Table 5-1

Summary of the analytical DEAE profiles of hyaluronan and proteoglycans following treatment of vascular SMCs with fenofibrate. Monkey vascular SMCs were treated with fenofibrate (30 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) and metabolically labelled with both [^{35}S]-sulfate and [^{3}H]-glucosamine. The data from Figure 5-3 and 5-4 were used to obtain the ratio of HA to proteoglycans (PGs), calculated by analyzing the area under each peak. Data represents 2 individual experiments with similar results (n=2 for calculation of HA:PG ratio). The sample number does not allow for proper statistical analysis of the data however, with more experimental data the changes in the HA:PG ratio following treatment of vascular SMCs with fenofibrate would be significantly different.

To broaden the analysis of hyaluronan synthesized by vascular SMCs treated with fenofibrate, we used a non-radioactive mass assay. Confluent monkey SMCs seeded on a 24-well plate in DMEM containing FBS (5%) and glucose (25 mmol/L), were serum deprived for 48 h followed by treatment with fenofibrate (30 μmol/L) or troglitazone (10 μmol/L) in the presence and absence of TGF-β1 (2 ng/mL) for 24 h. The culture medium was analyzed for hyaluronan using the HA-br colorimetric mass assay (Chapter 2, Section 2.13). The treatment of monkey SMCs with fenofibrate (30 μmol/L) reduced the amount of hyaluronan synthesized by 54.7% ($P<0.05$) compared to untreated cells (Figure 5-5). Stimulation of vascular SMCs with TGF-β1 increased the amount of hyaluronan synthesized from 1181 ± 194 ng/10^6 cells to 1517 ± 252 ng/10^6 cells (Figure 5-5). Fenofibrate treatment of vascular SMCs in the presence of TGF-β1 reduced the amount of hyaluronan by 48.4% ($P<0.05$) compared to cells treated with TGF-β1 alone (Figure 5-5). These data show that fenofibrate treatment of vascular SMCs reduces the amount of hyaluronan and is supported by the earlier observations of reduced hyaluronan separated on the analytical DEAE columns.

Similar to fenofibrate treatment, vascular SMCs treated with the PPAR-γ ligand, troglitazone, showed an increase in [^{3}H]-hexosamine incorporation into CPC precipitable material (Figure 5-2A). To determine whether or not the increase in [^{3}H]-hexosamine incorporation was associated with an increase in hyaluronan, monkey SMCs treated with troglitazone (10 μmol/L) were assessed for hyaluronan using the HA-br. As observed with fenofibrate treatment, vascular SMCs treated with troglitazone showed a 47.6% reduction in the amount of hyaluronan present compared to untreated cells (Figure 5-6). Vascular SMCs treated with troglitazone (10 μmol/L) in the presence of TGF-β1 showed a 21.6% reduction in hyaluronan present compared to cells treated with TGF-β1 alone (Figure 5-6). These data, together with the analysis of hyaluronan by analytical DEAE confirm that the increase in

[^{3}H]-hexosamine incorporation into CPC precipitable material from cells treated with PPAR ligands is not due to an increase in hyaluronan synthesis. The likely reason for the increase in [^{3}H]-hexosamine is an increase in the specific activity of the hexosamine pool. An accepted "in-direct" method for the assessment of changes in the specific activity has been to metabolically label cell cultures with both [^{3}H]-glucosamine and [^{35}S]-sulfate, digest the GAGs with chondroitinase ABC and separate the monosulfated disaccharides (Δdi4S and Δdi6S) by HPLC or TLC (Yanagishita *et al.*, 1989). We chose to adopt this approach to determine whether the PPAR ligands alter the specific activity of [^{3}H]-hexosamine, however to separate and isolate the disaccharides we used the relatively new technique of FACE (Calabro *et al.*, 2000a; Calabro *et al.*, 2000b).
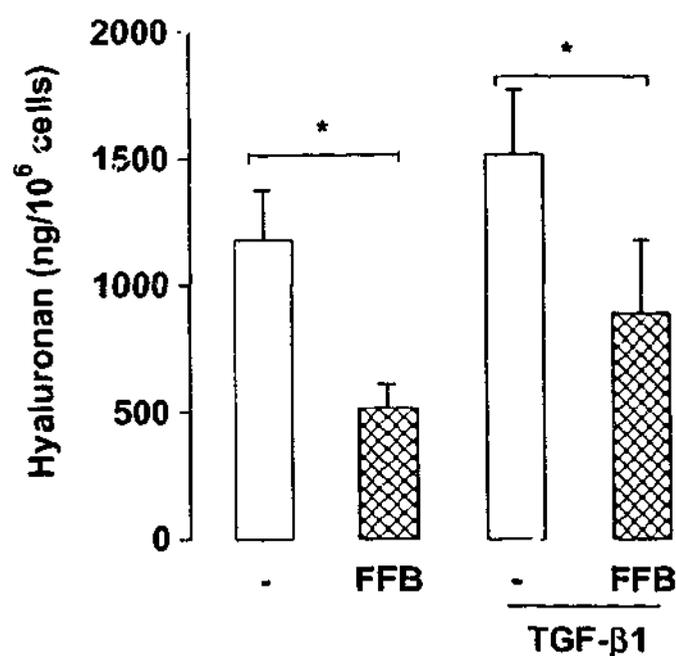
Figure 5-5

Fenofibrate treatment of vascular SMCs reduces hyaluronan in the presence and absence of TGF-β1. Monkey SMCs were treated with 0.1% DMSO (-) or fenofibrate (30 μmol/L; FFB) in the presence and absence of TGF-β1 (2 ng/mL). Hyaluronan secreted into the culture medium was assessed using the hyaluronan-binding region assay. These data represent the mean±SEM of 2 experiments performed in quadruplicate (n=12) and was analyzed using a 1-way ANOVA (*$P<0.05$).
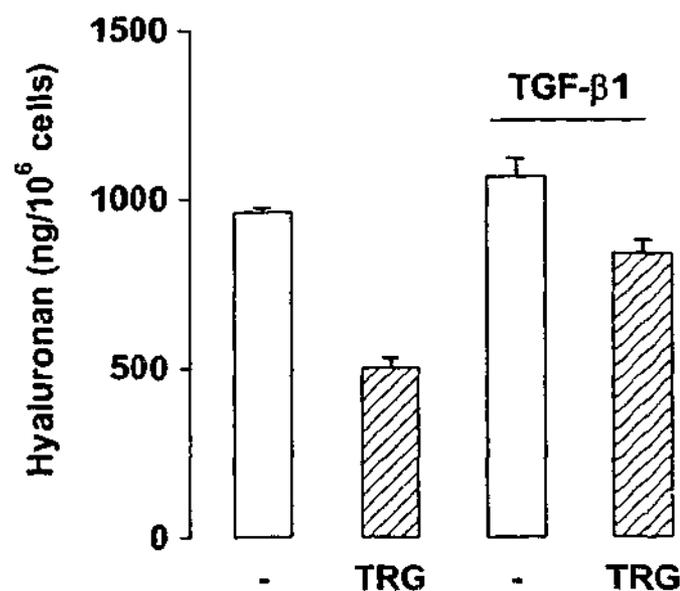
Figure 5-6

Troglitazone treatment of vascular SMCs reduces hyaluronan in the presence and absence of TGF-β1. Monkey SMCs were treated with 0.1% DMSO (-) or troglitazone (10 μmol/L; FFB) in the presence and absence of TGF-β1 (2 ng/mL). Hyaluronan secreted into the culture medium was assessed using the hyaluronan-binding region assay. These data represent the mean±SEM of 1 experiment performed in quadruplicate (n=4). The sample number does not allow for proper statistical analysis of the data however, with more experimental data the changes in hyaluronan following treatment of vascular SMCs with troglitazone would be significantly different.

5.2.3 The use of FACE to assess the change in specific activity of $[^3H]$-hexosamine following treatment of vascular SMCs with PPAR ligands

Quantitation of proteoglycans by the CPC precipitation assay showed that the incorporation of radiolabelled sulfate and glucosamine into proteoglycans gave apparently anomalous results. Since it has been well documented that there are experimental conditions which change the intracellular glucosamine pool (Yanagishita *et al.*, 1989; Yard *et al.*, 2002), we examined the possibility that the PPAR ligands were altering intracellular metabolism in a manner that affected the size and thus the specific activity of this glucosamine pool. The relative specific activity of the hexosamines was assessed by metabolic labelling of human vascular SMCs with both $[^{35}S]$-sulfate (50 µCi/mL) and $[^3H]$-glucosamine (10 µCi/mL). Secreted proteoglycans were digested with chondroitinase ABC. Disaccharides were fluorescently labelled with 2-aminoacridone (AMAC) and separated by FACE. After the fluorescent images were captured, the gel was held over UV light and the Δdi6S and Δdi4S bands were cut out of the gel using a scalpel blade. Each band was placed in a vial with scintillation fluid and counted for ^{35}S and ^3H in a Beckman Coulter liquid scintillation analyzer. The results were expressed as cpm (of radiolabel) per pixel, where pixel density is proportional to the mass of disaccharide because of the 1:1 stoichiometry of AMAC label to disaccharides (see Chapter 2, Figure 2-3).

The separation of radiolabelled and fluorescently-derivatized disaccharides by FACE showed that the major product (70-80%) was Δdi4S (Figure 5-7). The intensity and radiolabel incorporation into either Δdi6S or Δdi0S were close to background. Additionally there is no *a priori* reason that the labelling of the Δdi6S product would be different from the Δdi4S product most likely because they both use the same precursor pool. Hence, the analysis of specific activity for the Δdi4S product is presented.

Human vascular SMCs treated with fenofibrate (30 μmol/L) under low glucose conditions showed that the incorporation of $[^{35}S]$-sulfate into Δdi4S was not altered however treatment with troglitazone (10 μmol/L) reduced $[^{35}S]$-sulfate incorporation into Δdi4S by 20.9% compared to the low glucose control (Figure 5-8A). The effects of fenofibrate and troglitazone on $[^{35}S]$-sulfate incorporation into Δdi4S under low glucose conditions were similar to high glucose conditions (Figure 5-8A). These data support the results shown in Chapter 3, Section 3.2.10, that the treatment of vascular SMCs with fenofibrate does not alter the sulfation of GAGs. In contrast to fenofibrate, the treatment of vascular SMCs with troglitazone inhibits sulfation of disaccharides on proteoglycans.

Unlike the incorporation of $[^{35}S]$-sulfate into Δdi4S, the incorporation of $[^{3}H]$-galactosamine into Δdi4S was increased by 23.9% and 35.3% following treatment of vascular SMCs with fenofibrate and troglitazone respectively, under low glucose conditions (Figure 5-8B). The incorporation of $[^{3}H]$-galactosamine into Δdi4S was not altered by either of the PPAR ligands under high glucose conditions (Figure 5-8B). The treatment of vascular SMCs with fenofibrate in low glucose medium increased the ratio of ^{3}H to ^{35}S in Δdi4S by 18.4% ($P<0.05$) compared to untreated cells (Figure 5-8C). Vascular SMCs treated with troglitazone in low glucose medium showed an increase in the ratio of ^{3}H to ^{35}S in Δdi4S by 88.0% ($P<0.01$) compared to untreated cells (Figure 5-8C). In the presence of high (25 mmol/L) glucose medium, the ratio of ^{3}H to ^{35}S in Δdi4S was significantly reduced by 43.9% ($P<0.01$) compared to low (5.5 mmol/L) glucose medium, however the effect of troglitazone to increase the ^{3}H to ^{35}S ratio was maintained under high glucose conditions (Figure 5-8C).

In summary, proteoglycan disaccharides from fenofibrate treated SMCs, show an increase in the $[^{3}H]$ to $[^{35}S]$ labelling ratio which arises from an increase in the incorporation of

[^{3}H]-galactosamine. Proteoglycan disaccharides from troglitazone treated SMCs show an increase in the [^{3}H] to [^{35}S] labelling ratio which arises from both an increase in the incorporation of [^{3}H]-galactosamine and a decrease in the incorporation of [^{35}S]-sulfate. These data show that troglitazone and to a lesser extent, fenofibrate have an effect to increase the ratio of ^{3}H to ^{35}S into disaccharides from GAGs, synthesized by human vascular SMCs, suggesting that there is an increase in the specific activity of radiolabelled glucosamine.

The data from Figure 5-8 were used to calculate the specific activity of [^{3}H]-galactosamine in Δdi4S. The specific activity (sa) of sulfate in the medium was calculated to be ≈59 μCi/μmol (50 μCi of [^{35}S]-sulfate/0.847 μmol sulfate/mL). We used the equation previously described by Hascall and colleagues (Yanagishita *et al.*, 1989)

$$sa(H) = sa(S) \times lr$$

to determine the specific activity of galactosamine, where lr = ^{3}H/^{35}S-labeling ratio in the monosulfated Δdi4S following separation by FACE.

The results show that fenofibrate treatment of vascular SMCs increased the specific activity of [^{3}H]-galactosamine in Δdi4S from 48.2±3.2 μCi/μmol to 57.1±2.6 μCi/μmol ($P<0.05$) under low glucose conditions (Figure 5-9). Troglitazone treatment of vascular SMCs significantly increases the specific activity of [^{3}H]-galactosamine in Δdi4S from 48.2±3.2 μCi/μmol to 90.7±11.0 μCi/μmol ($P<0.01$), under low glucose conditions (Figure 5-9). The effect of fenofibrate and troglitazone to increase the specific activity of [^{3}H]-galactosamine into Δdi4S is dampened under high glucose conditions (Figure 5-9). To determine whether the change in the specific activity of [^{3}H]-hexosamine was due to an increase in glucose utilization through the glycolysis metabolic pathway with a consequential increase in lactate production, we tested the rate of glucose utilization and lactate production in human vascular SMCs following treatment with PPAR ligands.

Disaccharide
Standards

ΔdiHA>
Δdi0S>

Non-specific
fluorescent band>

Δdi6S>
Δdi4S>
ΔdiUA2S>

− FFB TRG − FFB TRG

5.5 mmol/L glucose 25 mmol/L glucose
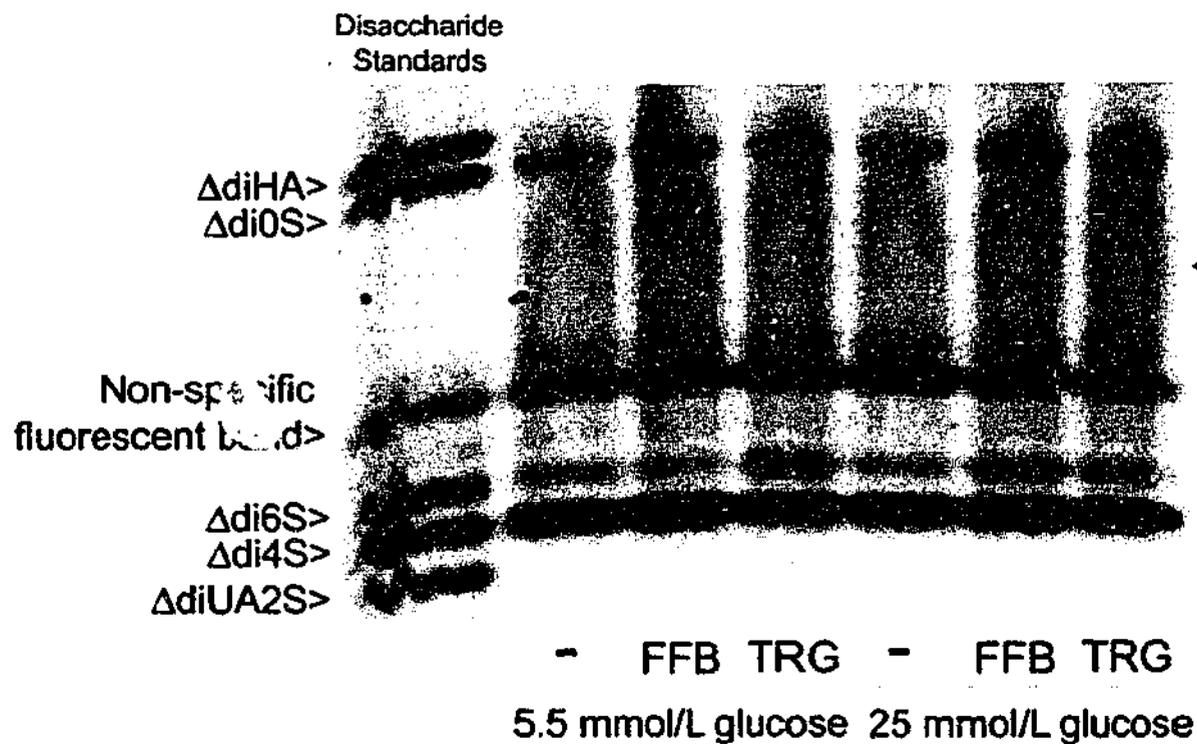
Figure 5-7

Separation of radiolabelled and fluorescently derivatized disaccharides by FACE.
Proteoglycans were isolated from human vascular SMCs treated with 0.1% DMSO (-),
fenofibrate (FFB; 30 μmol/L) or troglitazone (TRG; 10 μmol/L) and metabolically labelled
with both [^{35}S]-sulfate and [^{3}H]-glucosamine in culture medium containing either low
(5.5 mmol/L) or high (25 mmol/L) glucose. GAGs were digested with chondroitinase ABC,
disaccharides were fluorescently labelled with AMAC and separated by FACE. Gel represents
similar results from 2 experiments performed in duplicate.
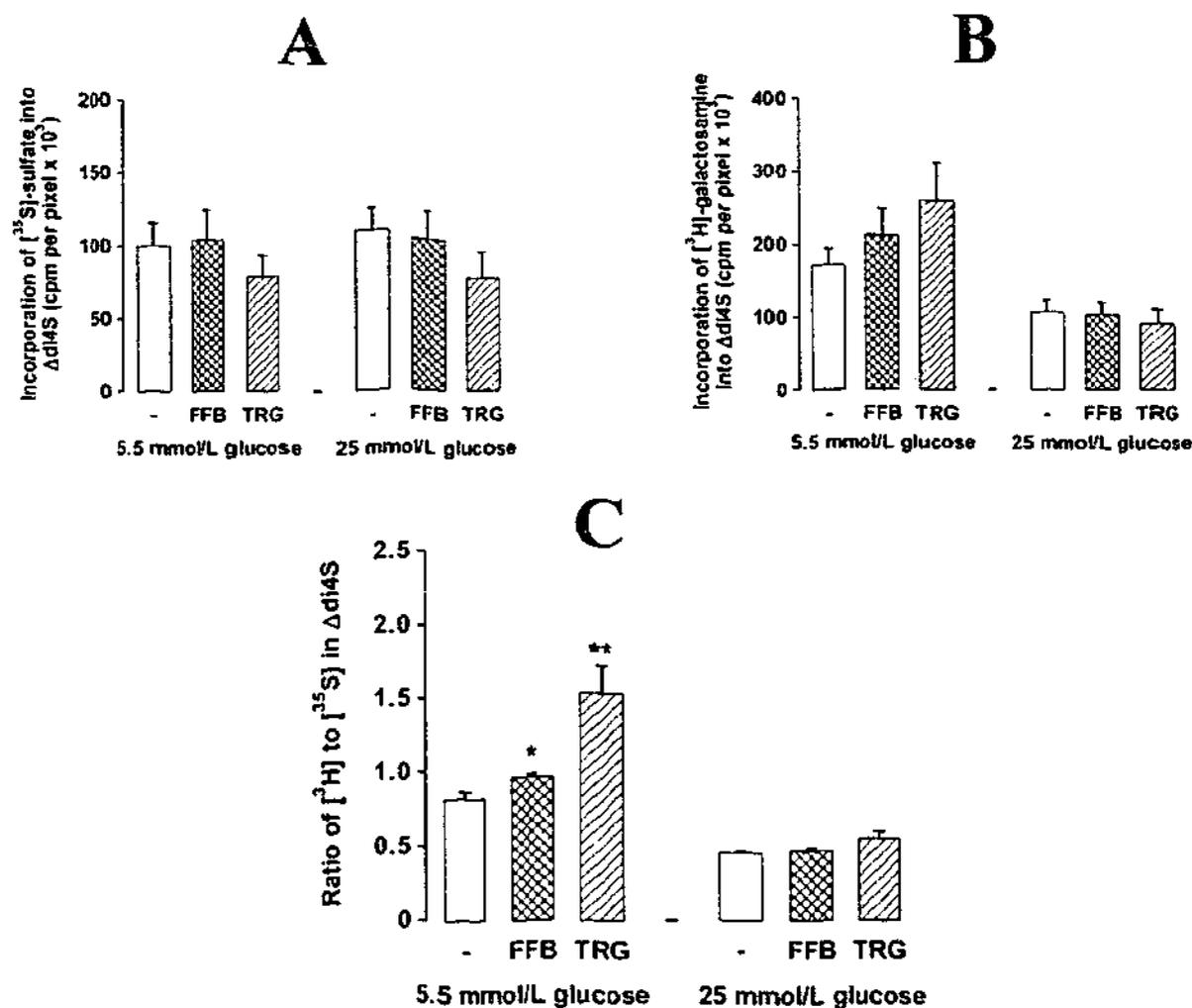
Figure 5-8

Treatment of human vascular SMCs with PPAR ligands, increases the ratio of [^{3}H]-galactosamine:[^{35}S]-sulfate in __Δdi4S__. Proteoglycans were isolated from human vascular SMCs treated with 0.1% DMSO (-), fenofibrate (FFB; 30 μmol/L) or troglitazone (TRG; 10 μmol/L) and metabolically labelled with both [^{35}S]-sulfate and [^{3}H]-glucosamine in culture medium containing either low (5.5 mmol/L) or high (25 mmol/L) glucose. GAGs were digested with chondroitinase ABC, disaccharides were fluorescently labelled with AMAC and separated by FACE (see Figure 5-7). The Δdi4S band was excised from the gel and assessed for [^{35}S] and [^{3}H]. (A) Incorporation of [^{35}S]-sulfate into Δdi4S and (B) incorporation of [^{3}H]-galactosamine incorporation into Δdi4S. (C) The ratio of ^{3}H/^{35}S for Δdi4S. Data (n=4) is expressed as the mean±SEM of 2 experiments performed in duplicate and was analyzed by a Student's t-test (* $P<0.05$, ** $P<0.01$, versus the low glucose control).
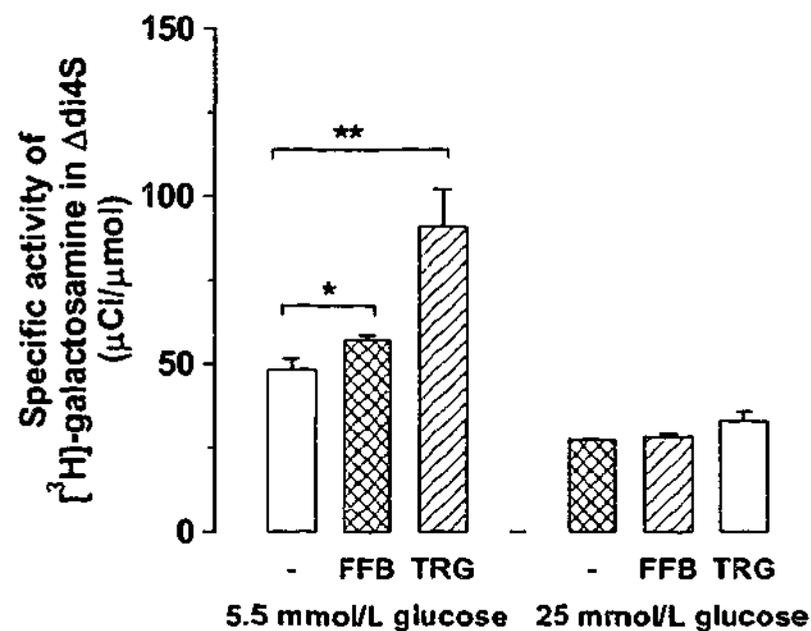
Figure 5-9

Treatment of human vascular SMCs with PPAR ligands, increases the specific activity of [³H]-galactosamine in Δdi4S of GAGs. The change in the specific activity of [³H]-galactosamine in the Δdi4S product following treatment of vascular SMCs with 0.1% DMSO (-), fenofibrate (FFB; 30 µmol/L) or troglitazone (TRG; 10 µmol/L) in culture medium containing either 5.5 mmol/L or 25 mmol/L glucose. The specific activity was calculated from the $^3H/^{35}S$-labelling ratio in the Δdi4S product following excision from the FACE gels. Data is expressed as the mean±SEM of 2 experiments performed in duplicate and was analyzed by a Student's t-test (* $P<0.05$, ** $P<0.01$ versus the low glucose control).

5.2.4   Effects of PPAR ligands on glucose metabolism

Vascular SMCs were treated with either fenofibrate (50 µmol/L) or troglitazone (10 µmol/L) in low (5.5 mmol/L) and high (25 mmol/L) glucose medium for 24 h. The culture medium was collected and measured for glucose consumption and lactate production using the Hexokinase/NADPH reaction and lactate oxidase and peroxidase respectively (Chapter 2, Section 2.14). The data indicates that under low glucose conditions, the amount of glucose consumed by vascular SMCs is 109.9 ± 23.5 nmol/L/hour/10^3 cells which is similar to the amount of glucose consumed by vascular SMCs maintained under high glucose conditions, 115.6 ± 17.4 nmol/L/hour/10^3 cells (Figure 5-10A). Lactate production by vascular SMCs growing in low and high glucose medium was 131.0 ± 25.4 nmol/L/hour/10^3 cells and 139.4 ± 20.1 nmol/L/hour/10^3 cells, respectively (Figure 5-10B). These results indicate that the amount of glucose entering the glycolysis pathway remains relatively constant regardless of the glucose concentration outside the cell at glucose concentrations at or above 5.5 mmol/L.

Vascular SMCs treated with fenofibrate (50 µmol/L) showed a modest 14.6% (not significant) increase in glucose consumption and 31.0% ($P<0.05$) increase in lactate production under low glucose conditions (Figure 5-10A and 5-10B). The lesser effect of fenofibrate treatment to increase glucose utilization but significantly increase lactate production may reflect the conversion of 1 mol glucose to 2 mol lactate. Fenofibrate treatment of vascular SMCs in high glucose medium does not affect glucose metabolism (Figure 5-10A and B). Troglitazone treatment of vascular SMCs increases glucose consumption 2-fold ($P<0.001$) under low glucose conditions and 2.5-fold ($P<0.001$) when the medium glucose concentration is 25 mmol/L (Figure 5-10A). Similarly, the treatment of vascular SMCs with troglitazone (10 µmol/L) in medium containing low (5 mmol/L) glucose, increased lactate production 2-fold ($P<0.001$) and in medium containing high (25 mmol/L) glucose, lactate

production increased 2.4-fold ($P<0.001$) (Figure 5-10B). These data indicate that the treatment of vascular SMCs with fenofibrate increases glucose metabolism under low glucose conditions, while troglitazone increases glucose metabolism under both low and high glucose conditions. The perturbation in glucose metabolism is associated with an alteration in the glucosamine pool, which is more apparent under low glucose conditions. Vascular SMCs treated with PPAR ligands under low glucose conditions, followed by metabolic labelling with [^{3}H]-glucosamine, increases the specific activity of the [^{3}H]-hexosamine precursor pool and this appears as an anomalous increase in proteoglycan synthesis when assessed for [^{3}H]-hexosamine incorporation into GAGs.
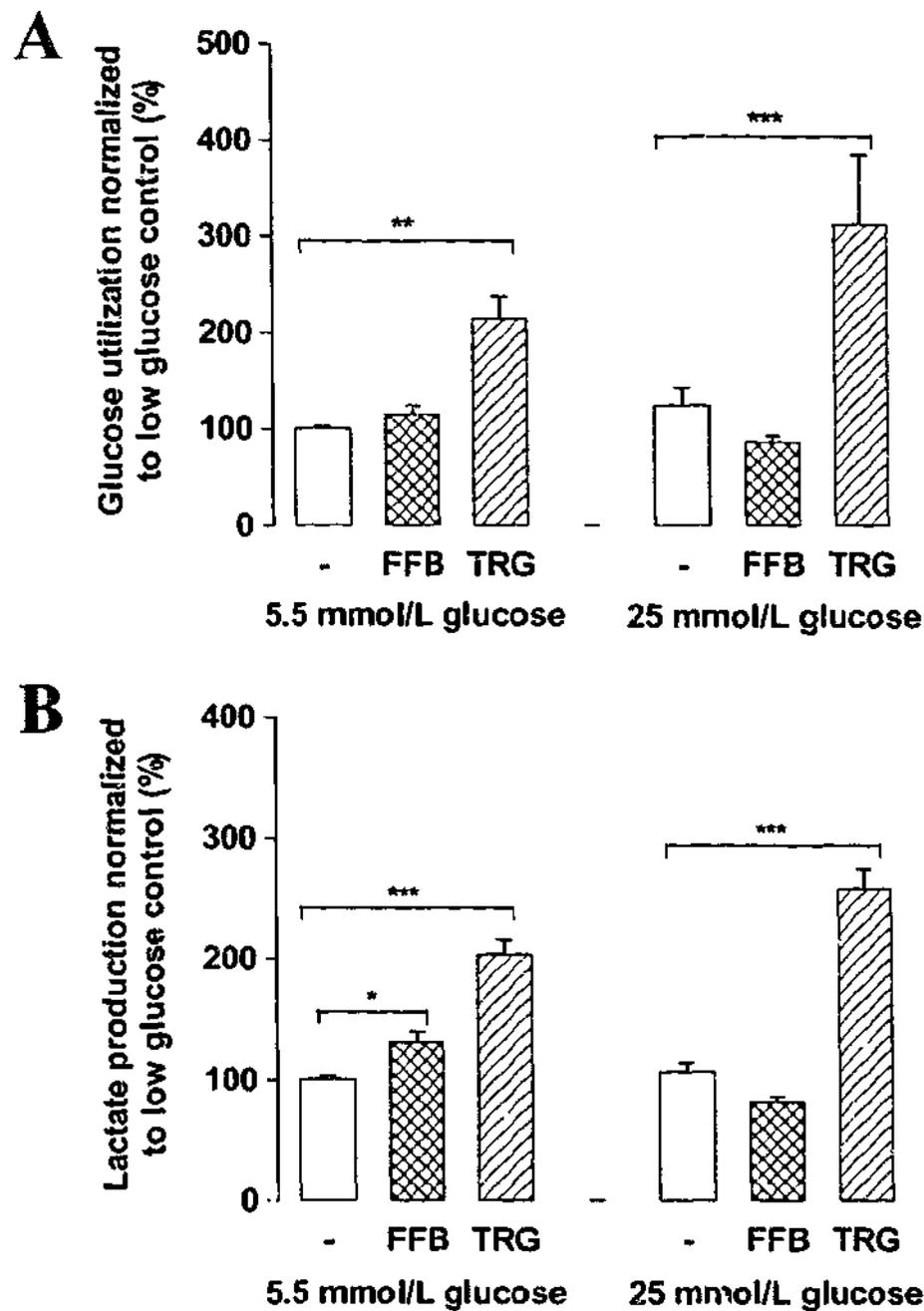
Figure 5-10

Treatment of vascular SMCs with PPAR ligands increases glucose metabolism. Human vascular SMCs were treated with 0.1% DMSO (-), fenofibrate (FFB; 50 μmol/L) or troglitazone (TRG; 10 μmol/L) under low (5.5 mmol/L) and high (25 mmol/L) glucose conditions for 24 h. The culture medium was collected and measured for (A) glucose utilization and (B) lactate production. Data is expressed as the mean±SEM of 3 experiments performed in triplicate and was analyzed by a 1-way ANOVA (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

5.3 Summary of results for Chapter 5

The analysis of the effect of fenofibrate treatment on vascular SMC proteoglycan synthesis using [^{3}H]-glucosamine in Chapter 3 (Section 3.2.6) provided the basis for the studies presented in Chapter 5. We have systematically addressed the possibilities for the anomalous increase in [^{3}H]-hexosamine incorporation into GAGs following the treatment of vascular SMCs with fenofibrate.

- Metabolic labelling of vascular SMCs with [^{3}H]-glucosamine following treatment with either fenofibrate, a PPAR-α ligand, or troglitazone, a PPAR-γ ligand, results in an increase in [^{3}H]-hexosamine incorporation into GAGs;

- The increase in [^{3}H]-hexosamine incorporation into GAGs was not associated with an increase in GAG length;

- The increase in [^{3}H]-hexosamine incorporation into GAGs is not related to an increase in hyaluronan synthesis because the treatment of vascular SMCs with fenofibrate decreased hyaluronan synthesis when assessed using two independent methods;

- Treatment of vascular SMCs with troglitazone decreased hyaluronan synthesis as assessed using a mass assay for hyaluronan;

- The increase in [^{3}H]-hexosamine incorporation into GAGs, following treatment of vascular SMCs with fenofibrate or troglitazone, was the result of an increase in the specific activity of radiolabelled glucosamine;

- Treatment of vascular SMCs with fenofibrate or troglitazone resulted in an increase in glucose utilization through the glycolysis pathway and thus altered the glucosamine precursor pool. This was observed as an anomalous increase in [^{3}H]-hexosamine incorporation into GAGs following metabolic labelling with [^{3}H]-glucosamine.

5.4 Discussion of results for Chapter 5

The PPAR-γ ligand, troglitazone has a strong effect to increase the specific activity of hexosamines incorporated into GAGs synthesized by human vascular SMCs and this is correlated with an increase in glycolysis (Figure 5-11). The PPAR-α ligand, fenofibrate has a weak effect to increase the specific activity of hexosamines and increase glycolysis (Figure 5-12). Under low glucose conditions the specific activity of the [^{3}H]-glucosamine was increased because the glucose concentration in the media limited the amount of glucosamine getting into the pool (Figure 5-11). Conversely, under high glucose conditions, treatment of vascular SMCs with troglitazone increases cellular glucose utilization, but since the glucose concentration in the media is high, the effect to alter the specific activity of [^{3}H]-glucosamine is lost because there is a dilution of the glucosamine pool, with glucose metabolic products (Figure 5-12). The mechanism for troglitazone to increase glucose utilization in vascular SMCs is most likely due to an increase in glucose uptake (Kihara et al., 1998; Yasunari et al., 1997) and the expression of the glucose transporter GLUT1 (Kihara et al., 1998) as has been previously described in these cells.

PPAR-α and –γ ligands have been shown (Nigro et al., 2002; Tannock et al., 2004) to reduce sulfate incorporation into proteoglycans in vascular SMCs which is associated with a reduction in the length of GAG chains. The incorporation of [^{3}H]-glucosamine into proteoglycans is increased following treatment of vascular SMCs with PPAR ligands and this is not a result of increased hyaluronan synthesis. Vascular SMCs treated with PPAR ligands and metabolically labelled with radiolabelled glucosamine alters the specific activity of the hexosamine pool as a result of increased glucose utilization. The changes in the specific activity of radiolabelled glucosamine following treatment of vascular SMCs with PPAR ligands does not affect the GAG shortening actions of these agents.

A

B

Low glucose conditions

Low glucose conditions + PPAR ligands
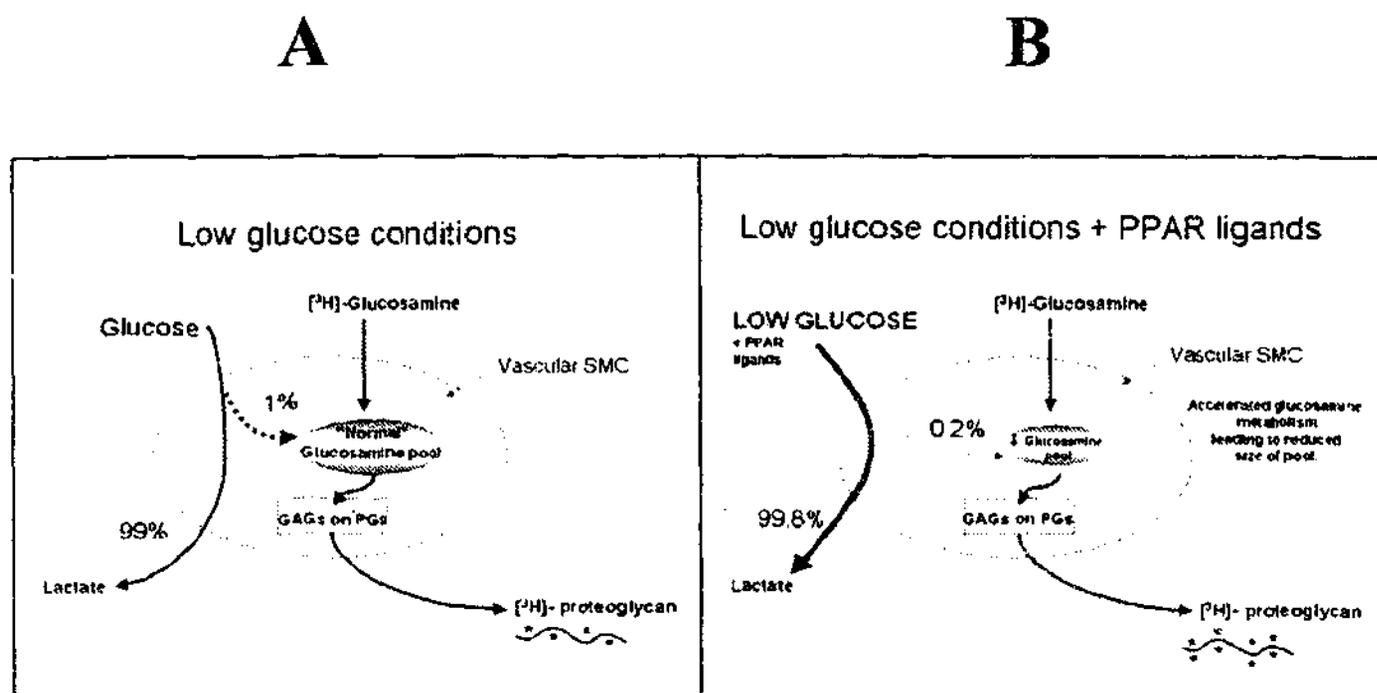
Figure 5-11

Illustrative representation of the changes in glucose and glucosamine metabolism in human vascular SMCs following treatment with PPAR ligands under low glucose conditions. (A) Under low glucose conditions, the typical proportions of glucose entering the glycolysis pathway and that which enters the hexosamine pathway are shown (exact percentages are illustrative and for explanation only). (B) Vascular SMCs which are in low (5.5 mmol/L) glucose medium and are treated with PPAR ligands, show increased glucose consumption and lactate production, which reduces the amount of glucose available for the glucosamine pool to about 0.2% (exact percentages are illustrative and for explanation only). The specific activity of radiolabelled glucosamine is increased resulting in increased incorporation of [^{3}H]-hexosamine into proteoglycans. * [^{3}H] label on proteoglycans.

A

High glucose conditions

HIGH GLUCOSE
[³H]-Glucosamine
Vascular SMC
10%
Diluted glucosamine pool
90%
GAGs on PGs
Lactate
[³H]- proteoglycan

B

High glucose conditions + PPAR ligands

HIGH GLUCOSE
+ PPAR ligands
[³H]-Glucosamine
Vascular SMC
4%
Diluted glucosamine pool
96%
GAGs on PGs
Lactate
[³H]- proteoglycan
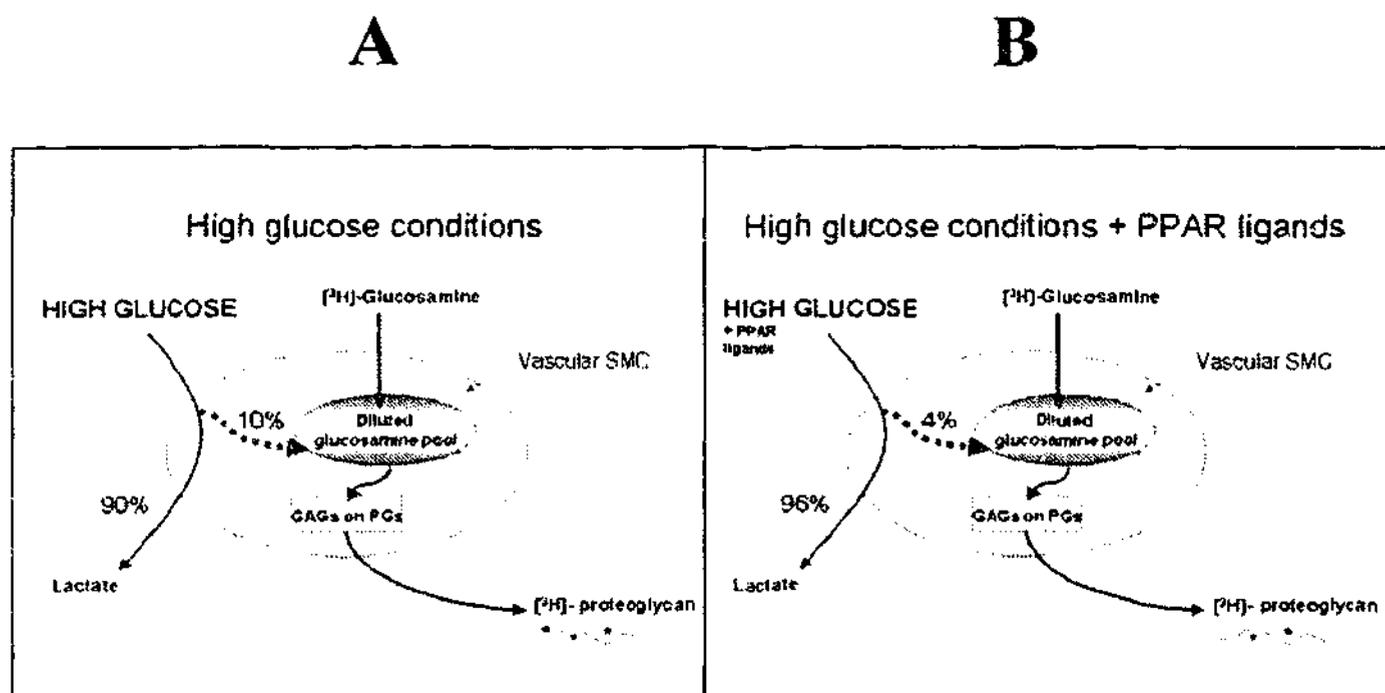
Figure 5-12

Illustrative representation of the changes in glucose and glucosamine metabolism in human vascular SMCs following treatment with PPAR ligands under high glucose conditions. (A) Under high glucose conditions, the typical proportions of glucose entering the glycolysis pathway and that which enters the hexosamine pathway are shown (exact percentages are illustrative and for explanation only). (B) Vascular SMCs which are in high (25 mmol/L) glucose medium and are treated with PPAR ligands, show increased glucose consumption and lactate production however, the concentration of glucose outside the cell is such that there is enough if not more glucose available to go through the hexosamine pathway (exact percentages are illustrative and for explanation only). Hence, the effect of PPAR ligands to increase the specific activity of radiolabelled glucosamine under low glucose conditions is not observed or is dampened under high glucose conditions (exact numbers are for explanation only). * [³H] label on proteoglycans.

Chapter 6: Growth factor, hormone and pharmacological regulation of proteoglycan biosynthesis in vascular smooth muscle

6.1   Introduction to results for Chapter 6

During the course of the project on fibrates, parallel studies were conducted with a variety of growth factors and pharmacological agents to explore relevant or related mechanisms to those which may apply to fibrates. Although not central to the action of fibrates, novel experiments and results are brought together and presented in this chapter. Most of the growth factors and pharmacological agents used in these studies have a known mechanism of action however the characterization of their effects to regulate proteoglycan synthesis (especially GAG length) is not complete in human vascular SMCs. We demonstrate that TGF-β1 and PDGF are potent stimulators of proteoglycan synthesis in human vascular SMCs while angiotensin II shows a weak stimulatory effect. Pharmacological regulation of proteoglycan synthesis is assessed by examining the alterations in proteoglycan GAG chain length using calcium channel antagonists and the sulfation pattern of the disaccharides which make up the GAGs using the sulfate inhibitor, sodium chlorate. Furthermore, our laboratory has identified a highly potent GAG elongation inhibitor (a protein tyrosine kinase inhibitor) and with the use of proteomics, this agent was used to explore possible enzymes that modify GAG length as these were the likely targets of fibrates.

6.2 Results of Chapter 6

6.2.1 Growth factor and hormone regulation of proteoglycan synthesis

6.2.1.1 Dose response curve for TGF-β1, PDGF and Angiotensin II – effect on vascular proteoglycan synthesis

Various growth factors are known to stimulate proteoglycan synthesis in vascular SMCs (Figueroa & Vijayagopal, 2002; Little et al., 2002; Schonherr et al., 1993; Schonherr et al., 1991) however the dose-response relationships in the human IMA SMCs used for the studies in this thesis have not been reported. To assess the range of growth factors which may activate proteoglycan biosynthesis in IMA smooth muscle, we examined platelet derived growth factor (PDGF), transforming growth factor (TGF)-β1 and angiotensin II (AII) being tyrosine kinase, serine-threonine and seven transmembrane G-coupled protein receptor (7TMGPR) agonists, respectively.

Merrilees et al. (Merrilees & Scott, 1990) have shown that the stimulation of porcine SMCs with TGF-β1 (0.01-2.0 ng/mL) results in a concentration-related increase in $[^3H]$-acetate incorporation into glycosaminoglycans suggesting increased synthesis. Additionally, this group showed that the treatment of porcine SMCs with 1000 pg/10^6 cells of TGF-β1 could stimulate proteoglycan synthesis, while a higher concentration (2600 pg/10^6 cells) could inhibit proteoglycan synthesis (Merrilees & Scott, 1990). We established concentration-response curves for TGF-β1, PDGF and AII in human vascular SMCs and assessed secreted proteoglycans by measuring $[^{35}S]$-sulfate incorporation into proteoglycans.

The response of vascular SMCs to TGF-β1 (0.01-3 ng/mL) has a "bell shaped" curve for proteoglycan synthesis, manifest as no stimulation at low concentration (<0.3 ng/mL) and inhibitory actions at high concentrations (>2 ng/mL, Figure 6-1). From this information it was decided that subsequent experiments would include TGF-β1 at a concentration of 1-2 ng/mL

for stimulating [^{35}S]-sulfate incorporation into proteoglycans and increasing the length of GAGs as presented in Chapter 3. These concentrations are within the peak of the curve. PDGF showed classical, sigmoidal, log-concentration response effects on proteoglycan synthesis in vascular SMCs, reaching a plateau at 100 ng/mL (Figure 6-2). For subsequent experiments, due to the cost of using PDGF, the effects of various agents on vascular proteoglycans in the presence of PDGF was studied at a concentration of 20-50 ng/mL, which is at the beginning of the plateau of the PDGF curve. Vascular proteoglycans synthesized in the presence of PDGF in combination with insulin (1 µmol/L), a model of the atherogenic and hyperinsulinemic environment of the vasculature in subjects with type 2 diabetes (Chait & Bierman, 1994), were used to study binding to LDL (see Chapter 4, Section 4.2.2). Angiotensin II gave a weak (maximum effect of 20%) stimulatory, sigmoidal concentration-response to proteoglycan synthesis in vascular SMCs, reaching a plateau at 30 nmol/L (Figure 6-3). The lack of response to AII may be due to the loss of AII receptors from multiply passaged cells (Bernstein & Berk, 1993; Gunther *et al.*, 1982). Loss of such receptors may be a consequence of altered SMC phenotype from contractile to secretory which occurs during passaging (Campbell & Campbell, 1993; Gunther *et al.*, 1982).
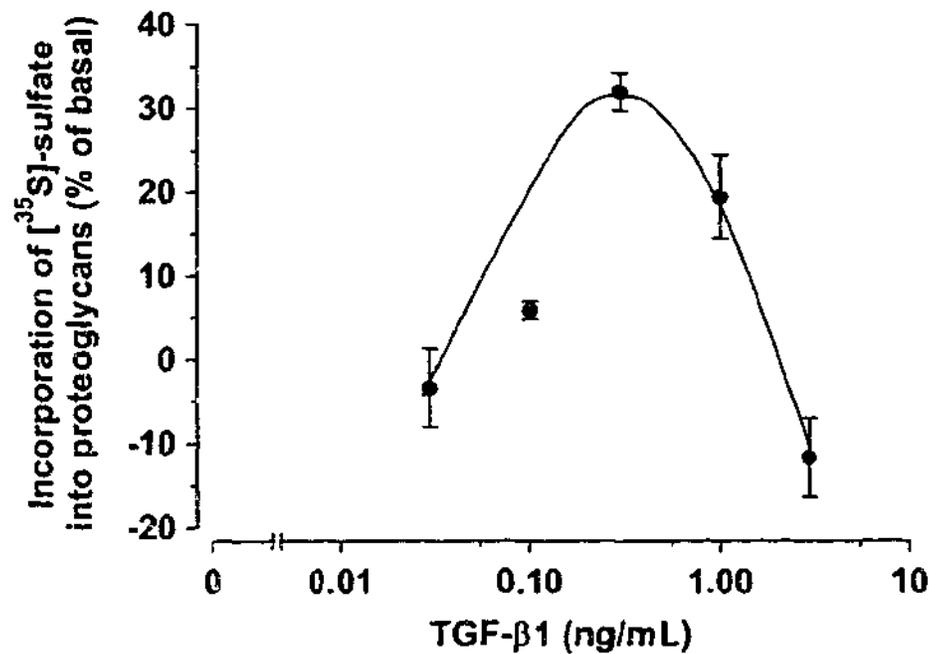
Figure 6-1

The concentration-response curve for the effect of TGF-β1 on proteoglycan synthesis in vascular SMCs is parabolic. The culture medium from vascular SMCs stimulated with increasing concentrations of TGF-β1 (0.03-3 ng/mL) was assessed for $[^{35}S]$-sulfate incorporation using the CPC precipitation assay. Each data point is the mean±SEM (n=6) of 2 experiments performed in triplicate. Basal value is 186.2±27.7cpm $[^{35}S]$-sulfate/10^3 cells. Because of the non-ideal nature of the data, a curved line was fitted manually.
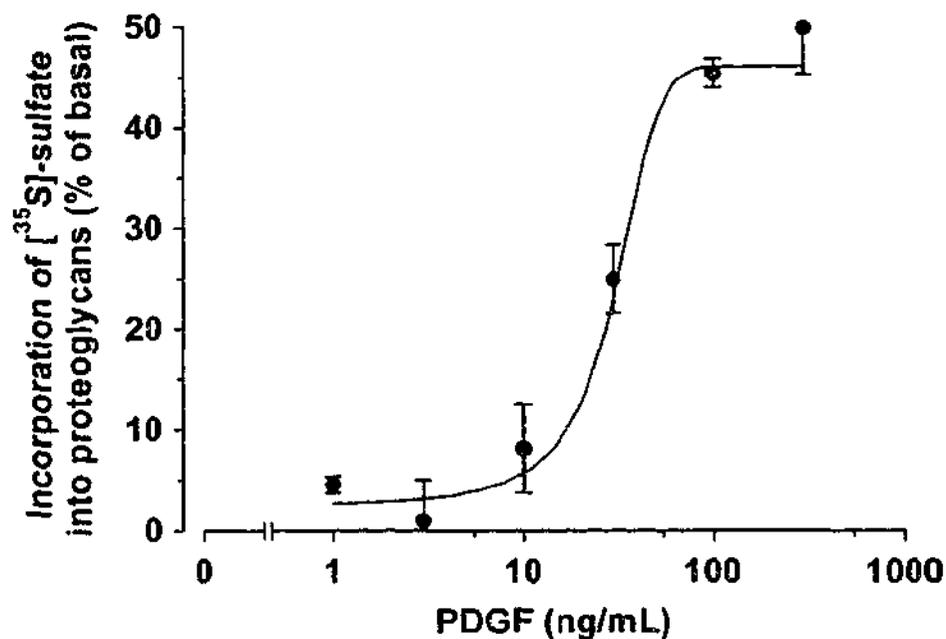
Figure 6-2

The concentration-response curve for the effect of PDGF on proteoglycan synthesis in vascular SMCs is logistic. The culture medium from vascular SMCs stimulated with increasing concentrations of PDGF (1-300 ng/mL) was assessed for $[^{35}S]$-sulfate incorporation using the CPC precipitation assay. Each data point is the mean±SEM (n=6) of 2 experiments performed in triplicate. Basal value is 185.9±34.0 cpm $[^{35}S]$-sulfate/10^3 cells. Data was fitted with a logistic sigmoid function in Fig.P version 2.98.
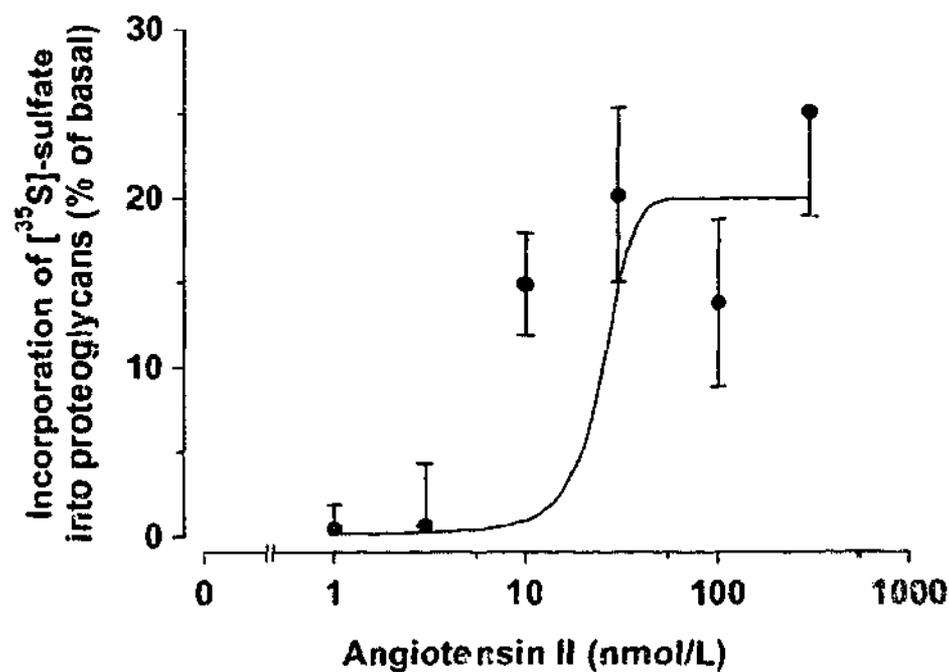
Figure 6-3

The concentration-response curve for the effect of angiotensin II on proteoglycan synthesis in vascular SMCs is logistic. The culture medium from vascular SMCs stimulated with increasing concentrations of Angiotensin II (1-300 nmol/L) was assessed for [^{35}S]-sulfate incorporation using the CPC precipitation assay. Each data point is the mean±SEM (n=6) of 2 experiments performed in triplicate. Basal value is 181.1±35.5 cpm [^{35}S]-sulfate/10^3 cells. Data was fitted with a logistic sigmoid function in Fig.P version 2.98.

6.2.1.2 The effect of TGF-β1 and PDGF on [³⁵S]-sulfate incorporation and GAG length is additive

To determine whether the effects of growth factors to increase proteoglycan synthesis and GAG length are synergistic or additive, we treated confluent, serum deprived human vascular SMCs with TGF-β1 (1 ng/mL), PDGF (50 ng/mL) and the two growth factors together and used metabolic labelling with [³⁵S]-sulfate. Following CPC precipitation analysis, the culture medium was applied to DEAE-Sepharose columns and proteoglycans were isolated by cold ethanol precipitation. Purified proteoglycans were separated by SDS-PAGE on 4-13% separating gel with a 3.5% stacking gel.

Vascular SMCs treated with TGF-β1 alone showed a 10.5% increase in [³⁵S]-sulfate incorporation into proteoglycans compared to cells maintained under basal conditions (Figure 6-4). Treatment of vascular SMCs with PDGF increased proteoglycan synthesis by 12.9% compared to untreated cells (Figure 6-4). Stimulation of vascular SMCs with both TGF-β1 and PDGF increased [³⁵S]-sulfate incorporation into proteoglycans by 27.1% compared to unstimulated cells, indicating that there is an additive effect of TGF-β1 and PDGF on proteoglycan biosynthesis (Figure 6-4). The electrophoretic mobility of vascular proteoglycans synthesized in the presence of TGF-β1 or PDGF alone is reduced, compared to proteoglycans from untreated SMCs (Figure 6-5). The treatment of vascular SMCs with both TGF-β1 and PDGF resulted in the synthesis of proteoglycans that showed a further reduction in electrophoretic mobility than proteoglycans from vascular SMCs treated with either growth factor alone (Figure 6-5). These data indicate that together, TGF-β1 and PDGF have an additive effect on GAG length. This suggests that the signalling pathways of TGF-β1 and PDGF controlling GAG length are distinct from each other such that when they are ' th used together, there is enhanced stimulation of either growth factor alone on human vascular SMC

proteoglycan synthesis.
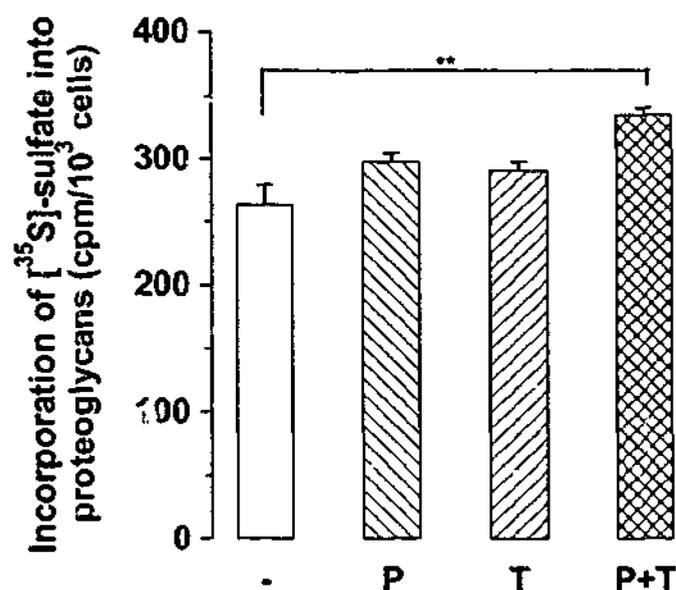
Figure 6-4

Stimulation of vascular SMCs with both TGF-β1 and PDGF has an additive effect to increase [^{35}S]-sulfate incorporation into proteoglycans. Incorporation of [^{35}S]-sulfate into vascular SMC proteoglycans, quantitated by the CPC precipitation assay following treatment with no growth factor (-), PDGF (P; 50 ng/mL), TGF-β1 (T; 1 ng/mL) or PDGF plus TGF-β1 (P+T; 50 ng/mL and 1 ng/mL respectively). Data are mean±SEM (n=3) of 1 experiment performed in triplicate and was analyzed using a 1-way ANOVA (** $P<0.01$).
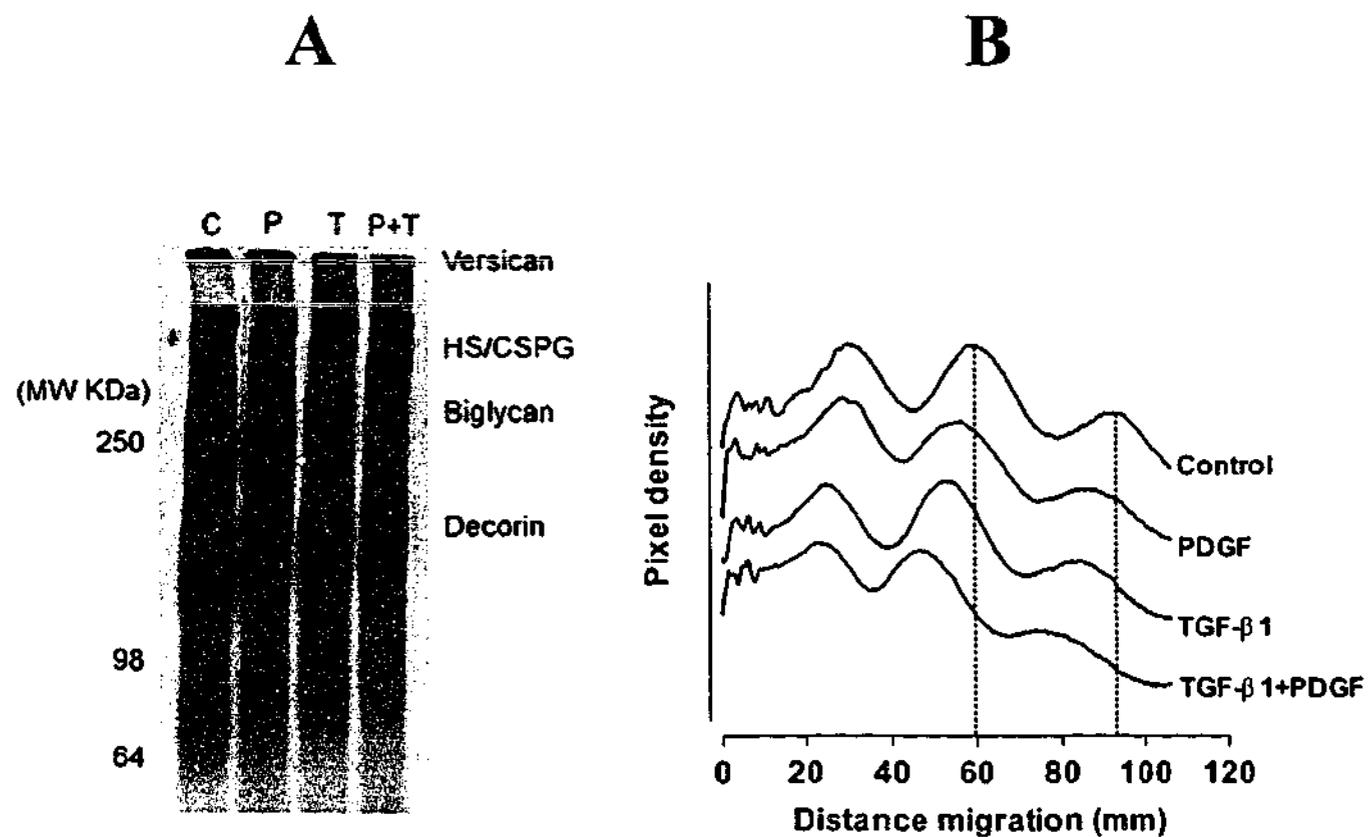
Figure 6-5

Stimulation of vascular SMCs with both TGF-β1 and PDGF has an additive effect to reduce the electrophoretic mobility of proteoglycans. (A) Proteoglycans, synthesized by vascular SMCs treated with no growth factors (C; Control), PDGF (P; 50 ng/mL), TGF-β1 (T; 1 ng/mL) and PDGF plus TGF-β1 (P+T; 50 ng/mL and 1 ng/mL respectively) were metabolically labelled with [^{35}S]-sulfate and separated by SDS-PAGE. (B) Densitometric scan of each lane from (A) not including the stacking gel; the dashed lines indicate the peaks of the proteoglycans from untreated cells. To facilitate data analysis, scans were strategically positioned to reflect the order of appearance on the gel using Fig.P Version 2.98.

6.2.2   Pharmacological regulation of proteoglycan synthesis

6.2.2.1   *Proteoglycan synthesis - the effect of calcium channel antagonists*

Calcium channel antagonists (CCAs) are used therapeutically as vasodilators in the treatment of dysrhythmia, angina and hypertension (Rang *et al.*, 1995). The CCAs are a chemically and pharmacologically diverse group of compounds however they all exert their primary vasodilatory effects on voltage-gated calcium channels. Voltage-operated calcium channels (VOCCs) open in response to membrane depolarization allowing Ca^{2+} ions to enter the cell. There are three distinct types of calcium channels; L, N and T (Tsien *et al.*, 1987). The L and T channels are the main calcium channels occurring in smooth muscle and cardiac muscle respectively, while the N channel is believed to be responsible for calcium entry into neurons (Rang *et al.*, 1995; Tsien *et al.*, 1987). Additionally, an increase in intracellular Ca^{2+} may act as a signal for calcium-receptor proteins (receptor-operated calcium channels; ROCCs), initiating cellular responses such as contraction, secretion, metabolic changes or the opening and closing of ion channels. The CCAs which act preferentially or solely on the L type channel are called dihydropyridine derivatives and include nifedipine, amlodipine and felodipine which are indicated for the treatment of hypertension. Verapamil is a cationic amphiphilic molecule, acts on both L- and T-type calcium channels and is used in treatment of arrhythmia and angina.

While the primary action of CCAs is to reduce blood pressure by L-type calcium channel blockade, there are reports of anti-atherogenic, "pleiotropic" actions independent of calcium channel blockade. For example, various CCAs have been shown to reduce the oxidative modification of LDL (Chen *et al.*, 1997; Mason *et al.*, 1999), and inhibit SMC migration (McMurray & Chahwala, 1992) and proliferation *in vitro* (Stepien *et al.*, 1998) and *in vivo* (Hirata *et al.*, 2000). A recent report has shown that cultured human vascular SMC

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proteoglycan synthesis, size and binding to LDL is reduced following treatment with moderate to high concentrations of calcium channel antagonists, nifedipine (14.4 µmol/L, 28.9 µmol/L and 57.8 µmol/L) and amlodipine (8.8 µmol/L, 17.6 µmol/L and 35.3 µmol/L) (Vijayagopal & Subramaniam, 2001). It was shown that these two dihydropyridine calcium antagonists decreased the size of the large chondroitin sulfate proteoglycan, versican in the absence of atherogenic growth factors (Vijayagopal & Subramaniam, 2001). These preliminary findings suggest that calcium may be involved in proteoglycan biosynthesis and possibly have actions on the signalling pathway(s) for proteoglycan synthesis.

In our study, we tested whether or not a dihydropyridine derivative (felodipine), a catamphilic CCA (verapamil) and a calcium agonist (Bay K 8644) have effects on proteoglycan synthesis in the presence of atherogenic growth factors, TGF-β1 and separately with PDGF. In vascular SMCs, VOCCs are blocked at low (nmol/L) concentrations of CCAs and ROCCs are blocked at much higher concentrations. In order to block all CCAs in vascular SMCs, we studied these agents at a concentration of 3 µmol/L. Vascular SMCs were metabolically labelled with [^{35}S]-sulfate for 24 h and the culture medium was assayed by CPC precipitation for total proteoglycans. Proteoglycans were isolated by ion-exchange chromatography and the electrophoretic mobility was assessed by SDS-PAGE.

Vascular SMCs treated with felodipine showed no effect on the incorporation of [^{35}S]-sulfate into proteoglycans under basal conditions, however treatment of vascular SMCs with verapamil and Bay K 8644 decreased [^{35}S]-sulfate incorporation into proteoglycans by 12.1% ($P<0.05$) and 15.2% ($P<0.01$), respectively (Figure 6-6). Stimulation of vascular SMCs with PDGF (50 ng/mL) increased [^{35}S]-sulfate incorporation into proteoglycans from 229.5±8.9 cpm/10^3 cells, under basal conditions to 301.2±3.6 cpm/10^3 cells (Figure 6-6). In the presence of PDGF, [^{35}S]-sulfate incorporation into proteoglycans was reduced by 15.9%

($P<0.01$), 29.3% ($P<0.001$) and 27.3% ($P<0.001$) following treatment of vascular SMCs with felodipine, verapamil and Bay K 8644, respectively compared to PDGF alone (Figure 6-6). Treatment of vascular SMCs with TGF-β1 (1 ng/mL) increased proteoglycan synthesis from 229.5±8.9 cpm/10^3 cells to 300.8 ±10.1 cpm/10^3 cells compared to cells maintained under basal conditions (Figure 6-6). In the presence of TGF-β1, vascular SMCs treated with felodipine, verapamil and Bay K 8644 showed a 17.7% ($P<0.001$), 19.2% ($P<0.001$) and 10.3% ($P<0.05$) reduction in sulfate incorporation into proteoglycans, respectively compared to TGF-β1 alone (Figure 6-6).

Further evaluation of proteoglycans from vascular SMCs treated with CCAs and a calcium channel agonist was performed using SDS-PAGE. The electrophoretic mobility of proteoglycans within the HS/CS band (Figure 6-7A and B) did not change following the treatment of vascular SMCs with felodipine, verapamil or Bay K 8644 compared to untreated cells. The electrophoretic mobility of the biglycan band from vascular SMCs treated with felodipine, verapamil or Bay K 8644 was increased compared to the biglycan band from untreated cells (Figure 6-7A and B). The treatment of vascular SMCs with the calcium agonist (Bay K 8644) but not the CCAs (felodipine or verapamil) resulted in the production of decorin with an increased electrophoretic migration compared to decorin from untreated cells (Figure 6-7A and B).

The electrophoretic migration of the HS/CS band was increased following the treatment of human vascular SMCs with felodipine, verapamil and Bay K 8644 in the presence of PDGF, compared to cells treated with PDGF alone (Figure 6-8A and B). The biglycan and decorin bands synthesized by vascular SMCs in the presence of PDGF showed a reduction in the electrophoretic mobility compared to biglycan and decorin from untreated cells, indicating that PDGF stimulates GAG elongation on these proteoglycans (Figure 6-8A and B). In the

presence of PDGF the treatment of vascular SMCs with felodipine, verapamil or Bay K 8644 did not change the electrophoretic mobility of biglycan or decorin compared to cells treated with PDGF alone (Figure 6-8A and B).

Vascular SMCs treated with TGF-β1 synthesized biglycan and decorin that showed a slower electrophoretic migration than biglycan and decorin from cells maintained under basal conditions (Figure 6-9A and B) supporting previous results shown in Chapter 3. The electrophoretic migration of the HS/CS band remained unchanged following the treatment of vascular SMCs with either felodipine, verapamil or Bay K 8644 in the presence of TGF-β1 (Figure 6-9A and B). The electrophoretic migration of the biglycan band was increased following the treatment of human vascular SMCs with felodipine in the presence of TGF-β1, compared to cells treated with TGF-β1 alone, however under these conditions verapamil and Bay K 8644 treatment showed no effect on the biglycan band (Figure 6-9A and B). Only the treatment of vascular SMCs with felodipine in the presence of TGF-β1 resulted in the synthesis of decorin with an increased electrophoretic mobility compared to decorin from cells treated with TGF-β1 alone (Figure 6-10A and B).

In summary, the treatment of vascular SMCs with felodipine resulted in a reduction in [^{35}S]-sulfate incorporation into proteoglycans which was associated with a consistent reduction in GAG length of biglycan and decorin, under the influence of atherogenic growth factors. A reduction in the GAG length of the HS/CS proteoglycan was also observed following the treatment of vascular SMCs with felodipine in the presence of PDGF. Treatment of vascular SMCs with verapamil or Bay K 8644 reduced [^{35}S]-sulfate incorporation into proteoglycans under all conditions however an apparent decrease in GAG length was only observed in the HS/CS proteoglycan following the treatment of vascular SMCs with either of these agents in the presence of PDGF. Unlike verapamil, Bay K 8644 reduced the GAG length

of decorin under basal conditions.
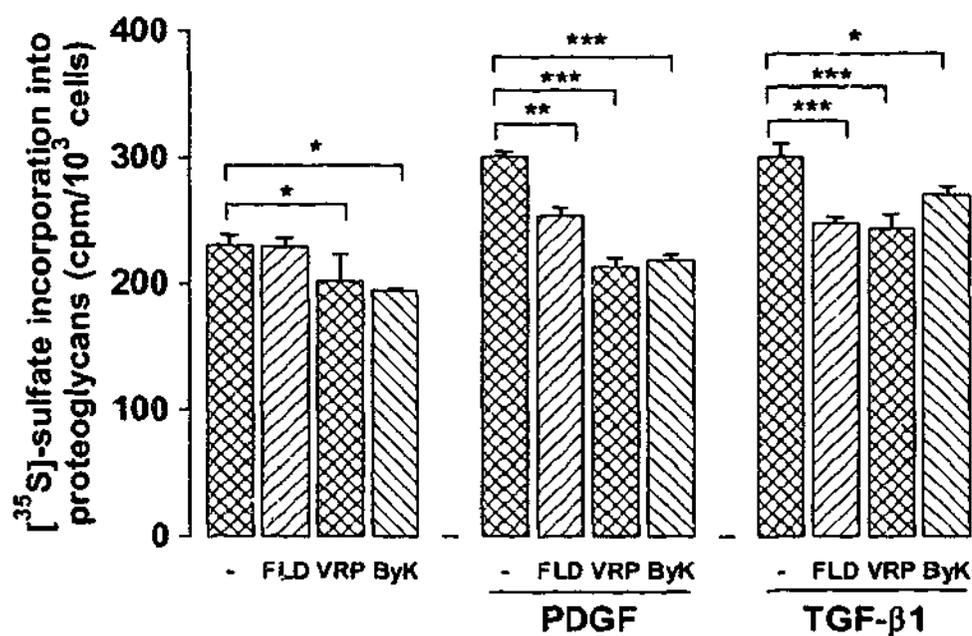
Figure 6-6

Proteoglycan synthesis is altered by the treatment of vascular SMCs with calcium channel antagonists, felodipine and verapamil and the calcium channel agonist, Bay K 8644. Human vascular SMCs were treated with 0.1% DMSO (-), felodipine (FLD, 3 μmol/L), verapamil (VRP, 3 μmol/L) and Bay K 8644 (ByK, 3 μmol/L) under basal conditions and in the presence of PDGF (50 ng/mL) or TGF-β1 (1 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Incorporation of [^{35}S]-sulfate into proteoglycans was measured by the CPC precipitation assay. Data are from 1 experiment performed in triplicate, are expressed as mean±SEM and were analyzed using a 1-way ANOVA (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

A   B
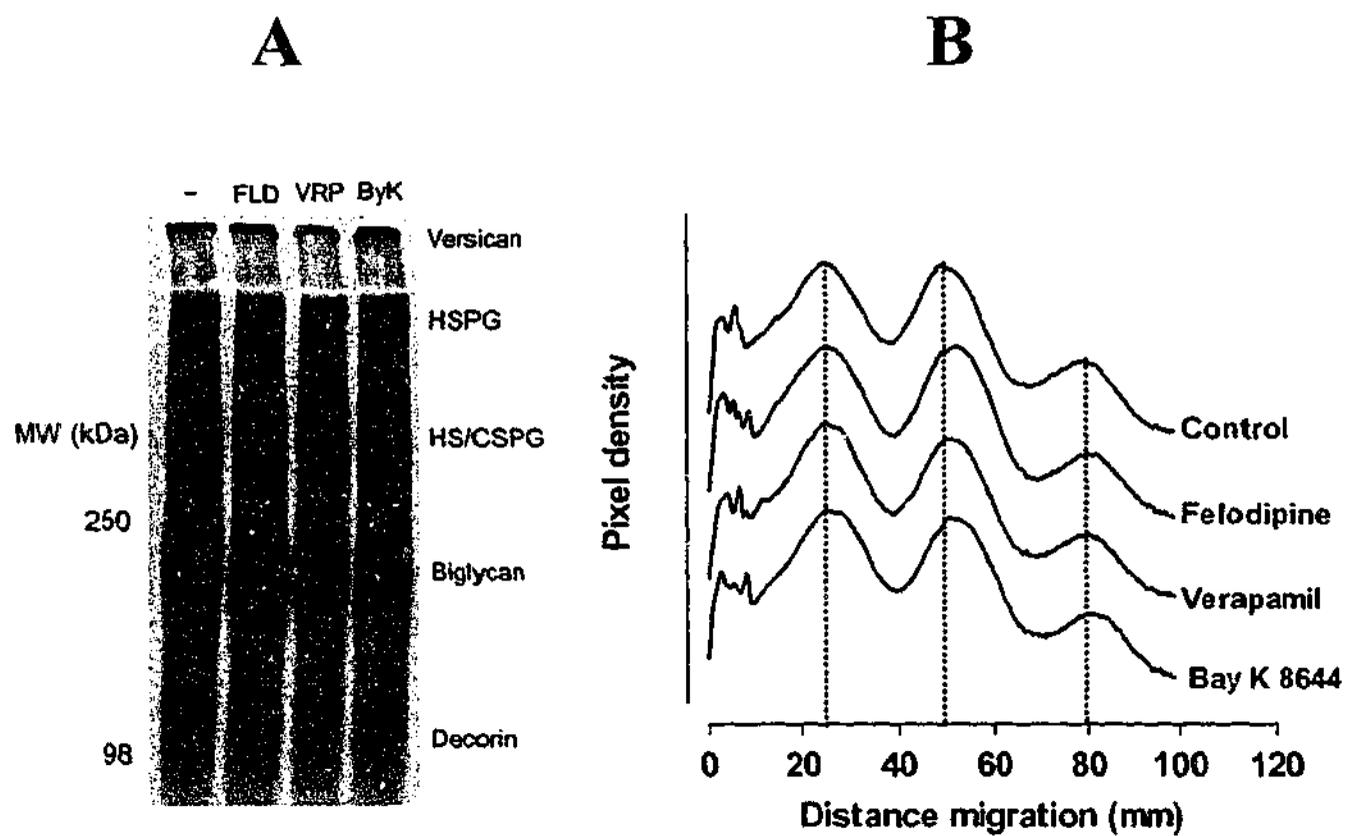
Figure 6-7

Vascular SMCs treated with calcium channel antagonists or a calcium channel agonist synthesize biglycan and decorin with increased electrophoretic mobility. (A) Vascular SMCs were treated with 0.1% DMSO (-; Control), felodipine (FLD, 3 μmol/L), verapamil (VRP, 3 μmol/L) or Bay K 8644 (ByK, 3 μmol/L) without growth factors and metabolically labelled with [^{35}S]-sulfate. Radiolabelled proteoglycans were separated on a 4-13% linear gradient separating gel with a 3.5% stacking gel. (B) Densitometric scan of each lane from (A) not including the stacking gel; the dashed lines indicate the peaks of the proteoglycans from untreated cells. To facilitate data analysis, scans were strategically positioned to reflect the order of appearance on the gel using Fig.P Version 2.98.
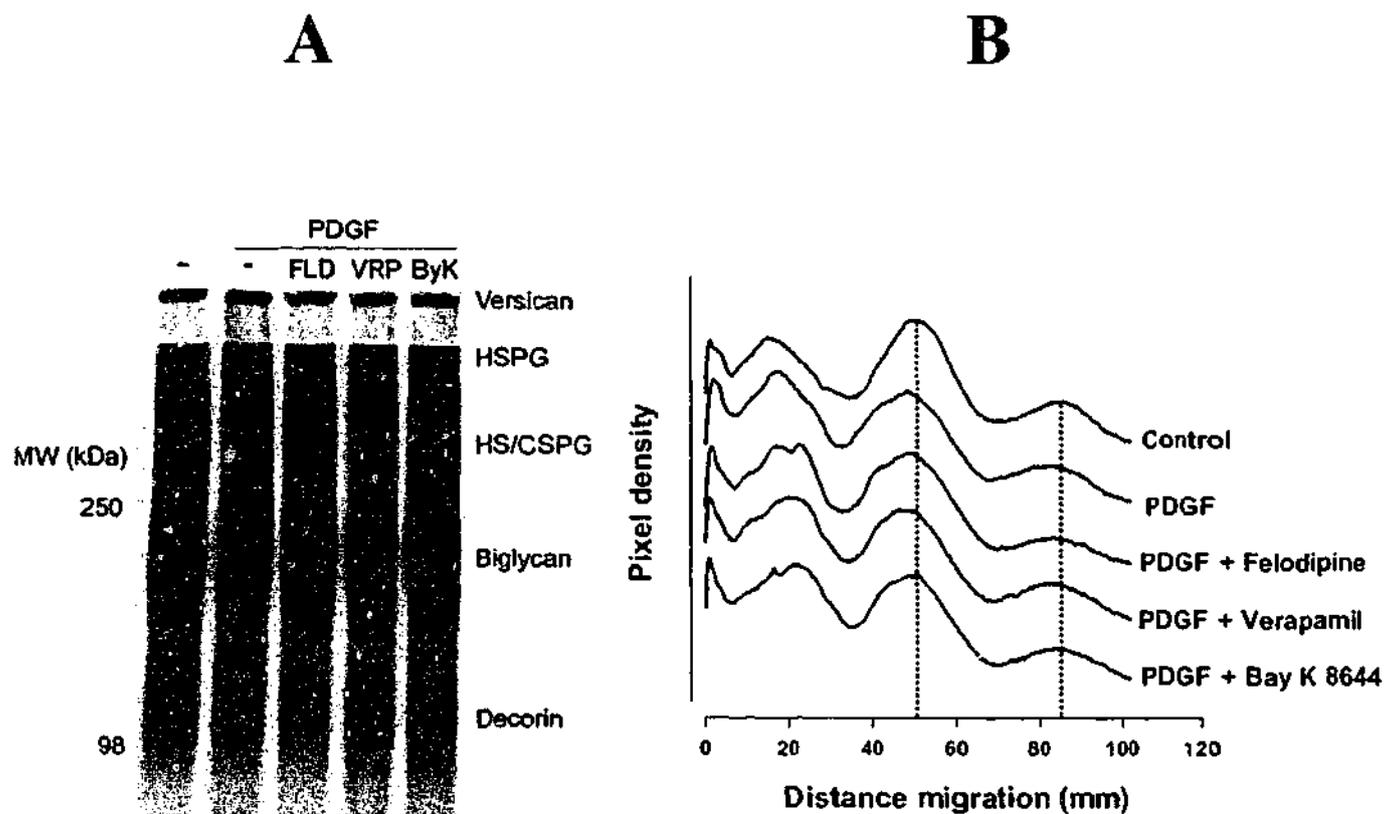
Figure 6-8

Increased electrophoretic mobility of HS/CS proteoglycan synthesized by vascular SMCs treated with calcium channel antagonists and a calcium channel agonist in the presence of PDGF. (A) Vascular SMCs were treated with 0.1% DMSO (-; Control), felodipine (FLD, 3 μmol/L), verapamil (VRP, 3 μmol/L) or Bay K 8644 (ByK, 3 μmol/L) in the presence of PDGF (50 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Radiolabelled proteoglycans were separated on a 4-13% linear gradient separating gel with a 3.5% stacking gel. (B) Densitometric scan of each lane from (A) not including the stacking gel; the dashed lines indicate the migration of biglycan and decorin from untreated cells. To facilitate data analysis, scans were strategically positioned to reflect the order of appearance on the gel using Fig.P Version 2.98.
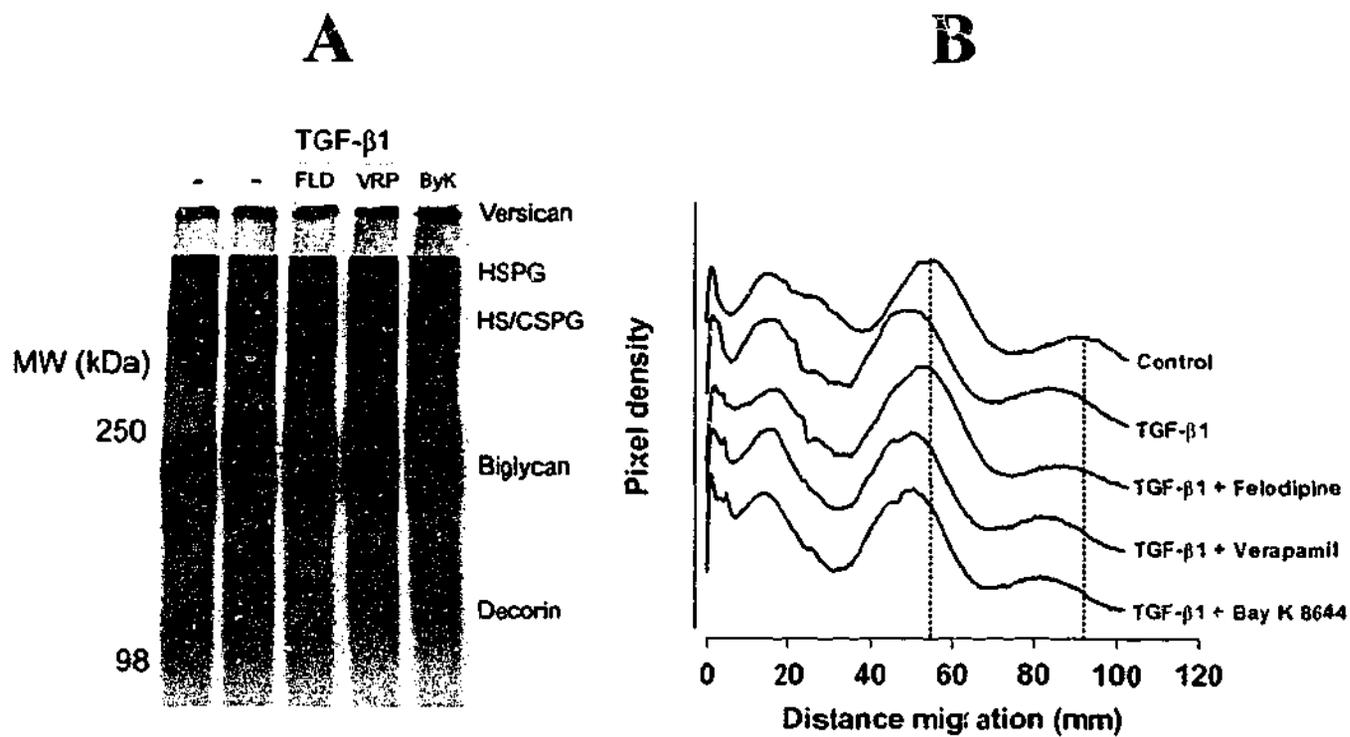
Figure 6-9

Biglycan from human vascular SMCs treated with felodipine in the presence of TGF-β1, has an increased electrophoretic mobility. (A) Vascular SMCs were treated with 0.1% DMSO (-; Control), felodipine (FLD, 3 μmol/L), verapamil (VRP, 3 μmol/L) or Bay K 8644 (ByK, 3 μmol/L) in the presence of TGF-β1 (1 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Radiolabelled proteoglycans were separated on a 4-13% linear gradient separating gel with a 3.5% stacking gel. (B) Densitometric scan of each lane from (A) not including the stacking gel; the dashed lines indicate the peaks of the proteoglycans from untreated cells. To facilitate data analysis, scans were strategically positioned to reflect order of appearance on the gel using Fig.P Version 2.98.

6.2.2.2   *Sulfation of disaccharides that form glycosaminoglycans – the effect of the sulfotransferase inhibitor, sodium chlorate*

Glycosaminoglycan degrading enzymes used to characterize the GAG chains associated with proteoglycans secreted by human vascular SMCs showed that chondroitin sulfate/dermatan sulfate GAGs were the predominant type of chains produced (Chapter 3, Section 3.2.11). Most of the CS/DS chains are composed of 4-sulfated *N*-acetylgalactosamine residues, with a lesser amount of 6-sulfated disaccharides and a very small amount of unsulfated disaccharides (Chapter 3, Section 3.2.10). The sulfation pattern of GAGs may influence binding to LDL (Gigli *et al.*, 1993) and *in vitro* studies have shown that the treatment of vascular SMCs with an atherogenic growth factor, such as PDGF increases the 6S to 4S ratio of the GAG chains (Schonherr *et al.*, 1993). Sodium chlorate has been characterized as a sulfotransferase inhibitor in human fibroblasts with a greater effect to reduce 4-sulfation than 6-sulfation (Greve *et al.*, 1988). A similar effect of sodium chlorate (0.3-3 mmol/L) treatment in vascular SMCs has recently been reported by our group (Ballinger *et al.*, 2004). The differential effect on 4- and 6-sulfation allows for an evaluation of the temporal sequence of events in the synthesis of GAGs. To demonstrate whether or not the FACE technique was capable of detecting changes in the sulfation pattern of CS/DS derived disaccharides induced by pharmacological agents such as fibrates, human vascular SMCs were treated with sodium chlorate (0.5-5 mmol/L).

Sodium chlorate treatment of vascular SMCs did not alter the 6-sulfated product but caused a concentration-dependent decrease in the Δdi4S which was accompanied by a concentration-dependent increase in the Δdi0S (Figure 6-10). These findings indicate that the mechanism of GAG synthesis in vascular SMCs is similar to GAG synthesis in other cells (Greve *et al.*, 1988) and further demonstrate that the FACE technique is suitable for detecting

and analyzing potential pharmacologically induced changes in the sulfation pattern of GAGs. We therefore used FACE to analyze the GAGs that had been synthesized by vascular SMCs in the presence of fenofibrate and troglitazone (see Chapters 3, Section 3.2.10 and Chapter 5, Section 5.2.3).
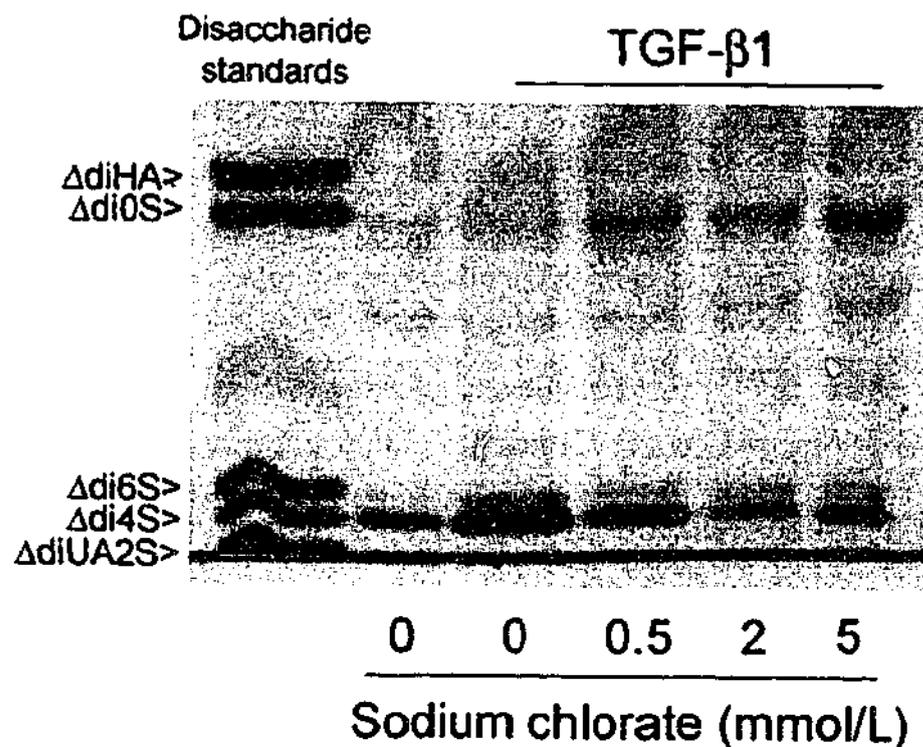
Figure 6-10

Validation of the Fluorophore Assisted Carbohydrate Electrophoresis (FACE) technique for assessing sulfation in human vascular SMCs. Fluorescent-tagged disaccharides from GAGs synthesized by vascular SMCs treated with sodium chlorate (0.5-5 mmol/L), were separated by FACE. Sodium chlorate (0.5-5 mmol/L) increases $\Delta di0S$ in a concentration-dependent manner. Sodium chlorate has a minimal effect on 6-sulfation. Sodium chlorate (0.5-5 mmol/L) inhibits 4-sulfation in a concentration-dependent manner. FACE gel is a representative of 2 individual experiments.

6.2.2.3 *A proteomics approach for a "blue-sky" search for proteins involved in the regulation of glycosaminoglycan length – the use of a protein tyrosine kinase inhibitor as an investigative tool*

Regulation of proteoglycan core protein mRNA with the broad tyrosine kinase inhibitor, genistein, has been previously reported in primate vascular SMCs, however this agent does not alter GAG length (Schonherr *et al.*, 1997) (Little *et al*, unpublished observations). Little *et al.* have identified a different protein tyrosine kinase inhibitor (KI) that is a potent and highly efficacious inhibitor of GAG elongation (Little *et al.*, unpublished observations). The nature of the compound is commercially sensitive but this does not detract from the situation that the current project had access to a potent and efficacious GAG elongation inhibitor to search for target enzymes. It is appreciated that the mechanism of action and hence, target for KI, may not be identical to fenofibrate.

The treatment of human vascular SMCs with KI in the presence and absence of PDGF results in the synthesis of proteoglycans with greatly reduced GAG length compared to proteoglycans from SMCs treated with PDGF alone (Figure 6-11A and B). We thus used proteomics and the treatment of vascular SMCs with KI as a tool to search for target protein(s) which may be involved in GAG elongation such as monosaccharide transporters or GAG elongation enzymes. To eliminate the multitude of tyrosine kinase regulated proteins that are not associated with GAG elongation, we used "differential pharmacology" by treating human vascular SMCs with either PDGF and genistein or PDGF/genistein and KI. In this way we assumed that the "classical" tyrosine kinase pathways inhibited by genistein (e.g. PDGF receptor) would overlap with the pathways inhibited by KI. We also assumed that the pathways inhibited by KI relating to GAG elongation would not overlap with the pathways of genistein because treatment of vascular SMCs with genistein does not affect GAG elongation

(Little *et al*, unpublished observations). We hypothesized that the proteins isolated from vascular SMCs treated with PDGF/genistein/K1 may show a different pattern of expression by 1-dimensional or 2-dimensional gel electrophoresis (refer to Chapter 2, Section 2.15) to proteins from vascular SMCs treated with PDGF/genistein and hence, we may identify critical proteins involved in GAG elongation.

A

PDGF

$-$ KI $-$ KI

MW (kDa)

250

98

HS/CS PG

Biglycan

Decorin

B

Pixel density

Con

Kinase inhibitor

PDGF

PDGF + kinase inhibitor

0   5   10   15   20   25

Distance migration (mm)
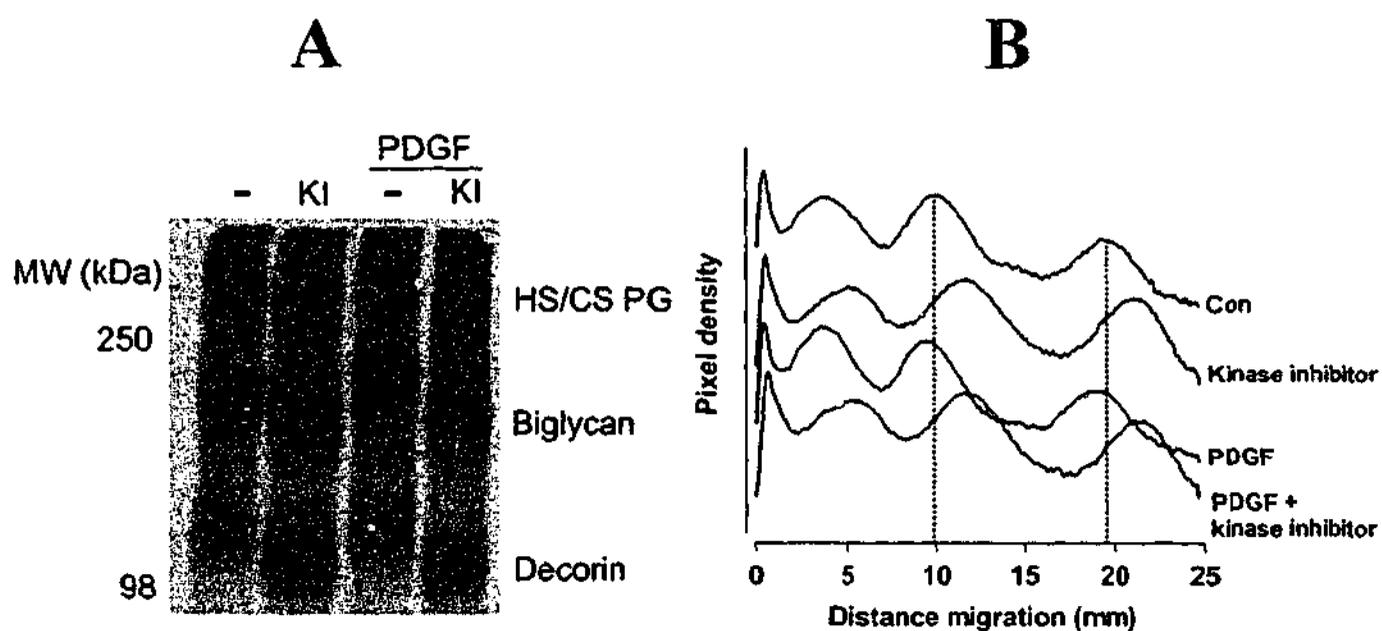
Figure 6-11

Increased electrophoretic mobility of proteoglycans synthesized by human vascular SMCs treated with a specific tyrosine kinase inhibitor. (A) Human vascular SMCs were treated with 0.1% DMSO (-; Con) or kinase inhibitor (KI, 1 µmol/L) in the presence and absence of PDGF (50 ng/mL) and metabolically labelled with [^{35}S]-sulfate. Radiolabelled proteoglycans were isolated and separated on a 4-20% SDS-PAGE. (B) The density profile of each lane from the gel presented in A. Dotted lines are the peak migration of biglycan and decorin from untreated SMCs. To facilitate data analysis, scans were strategically positioned to reflect order of appearance on the gel using Fig.P Version 2.98.

Protein from human vascular SMCs treated with PDGF/genistein or PDGF/genistein/KI for 6 h or 24 h was isolated and separated by 1-dimensional SDS-PAGE (Figure 6-12). The data showed that there was no change in the expression of isolated proteins between the two treatments (Figure 6-12). To increase the sensitivity of the search for regulated proteins, we used 2-dimensional gel electrophoresis. We found 4 proteins (spot no. 2, 3, 5, 6) from cells treated with PDGF/genistein that were not seen in the cellular protein from cells treated with PDGF/genistein/KI (Figure 6-13 and 6-14). Protein spot numbers 1 and 4 showed higher levels in cells treated with PDGF/genistein than cells treated with PDGF/genistein/KI (Figure 6-13 and 6-14). There was one protein (spot no. 7) from cells treated with PDGF/genistein/KI that was not detected in cells treated with PDGF/genistein (Figure 6-13 and 6-14). The numbered proteins were extracted, analyzed by mass spectroscopy and identified using MS-Fit and the results are summarized in Table 6-1. A database search identified spot number 1, of which there was more in cells treated with PDGF/genistein than cells treated with PDGF/genistein /KI, as a major histocompatibility complex (MHC) class I antigen. Spot number 3 was detected in cells treated with PDGF/genistein but not in cells treated with PDGF/genistein/KI and was matched to a Toll/interleukin-1 receptor domain-containing adapter protein with 16% coverage and a Ras-related protein, Rab-6B with 18% coverage. Protein spot number 5 was only detected in cells treated with PDGF/genistein and matched to a calcium/calmodulin-dependent protein kinase type II delta chain. Protein spots numbered 2, 4, 6 and 7 showed no matches in the database.
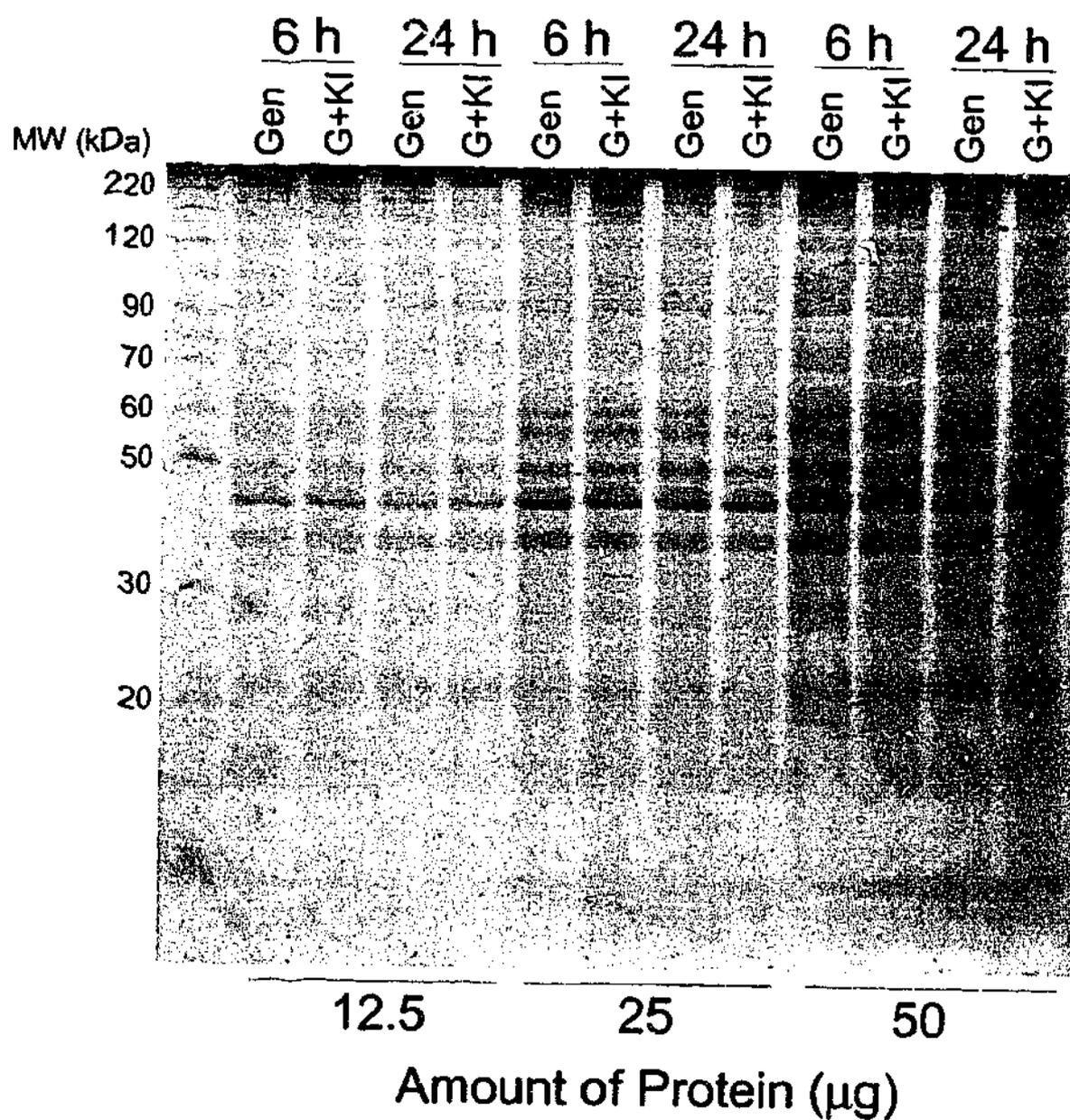
Figure 6-12

One-dimensional SDS-PAGE separation of proteins from vascular SMCs treated with PDGF/genistein in the presence and absence of KI. Protein was isolated from human vascular SMCs treated with PDGF/genistein (Gen; 50 ng/mL and 100 µmol/L respectively) or PDGF, genistein and KI (G+KI; 50 ng/mL, 100 µmol/L and 1 µmol/L, respectively) for 6 h or 24 h. Protein (12.5-50 µg) was separated on a 12.5% separating gel and stained with Coomassie Blue.

MW (kDa)
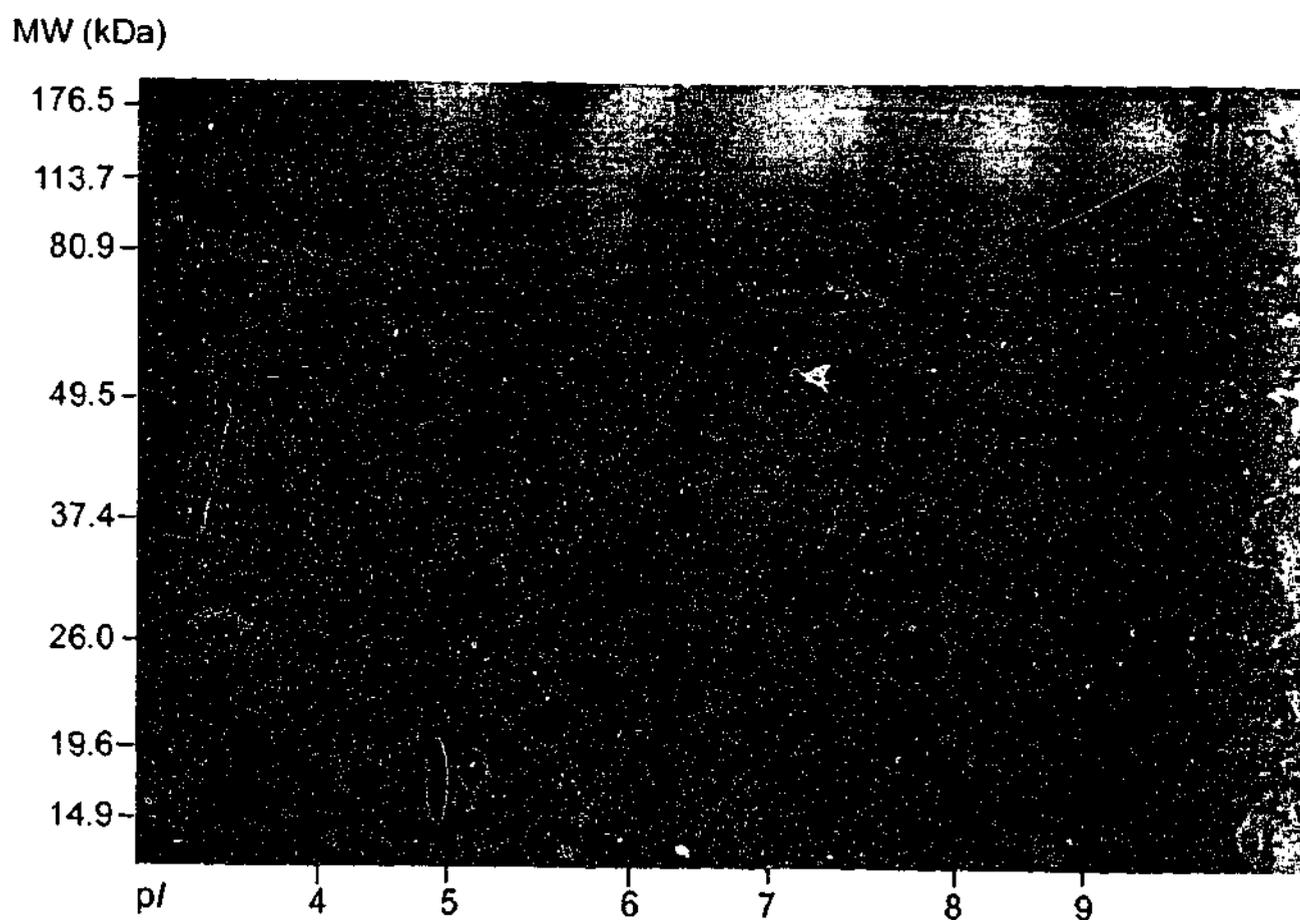
Figure 6-13

Two-dimensional gel separation of cellular proteins from human vascular SMCs treated with PDGF and genistein. Protein was isolated from human vascular SMCs treated with PDGF (50 ng/mL) and genistein (100 µmol/L) for 24 h. Protein (500 µg) was separated on a pH 3-10 strip followed by molecular weight separation on a 12.5% separating gel and stained with Coomassie Blue at 60°C. Red circled protein spots were detected only in cells treated with PDGF/genistein; blue circled spots had higher levels in cells treated with PDGF/genistein compared to cells treated with PDGF/genistein/K1. Spots were arbitrarily assigned a number from 1 to 6.

MW (kDa)
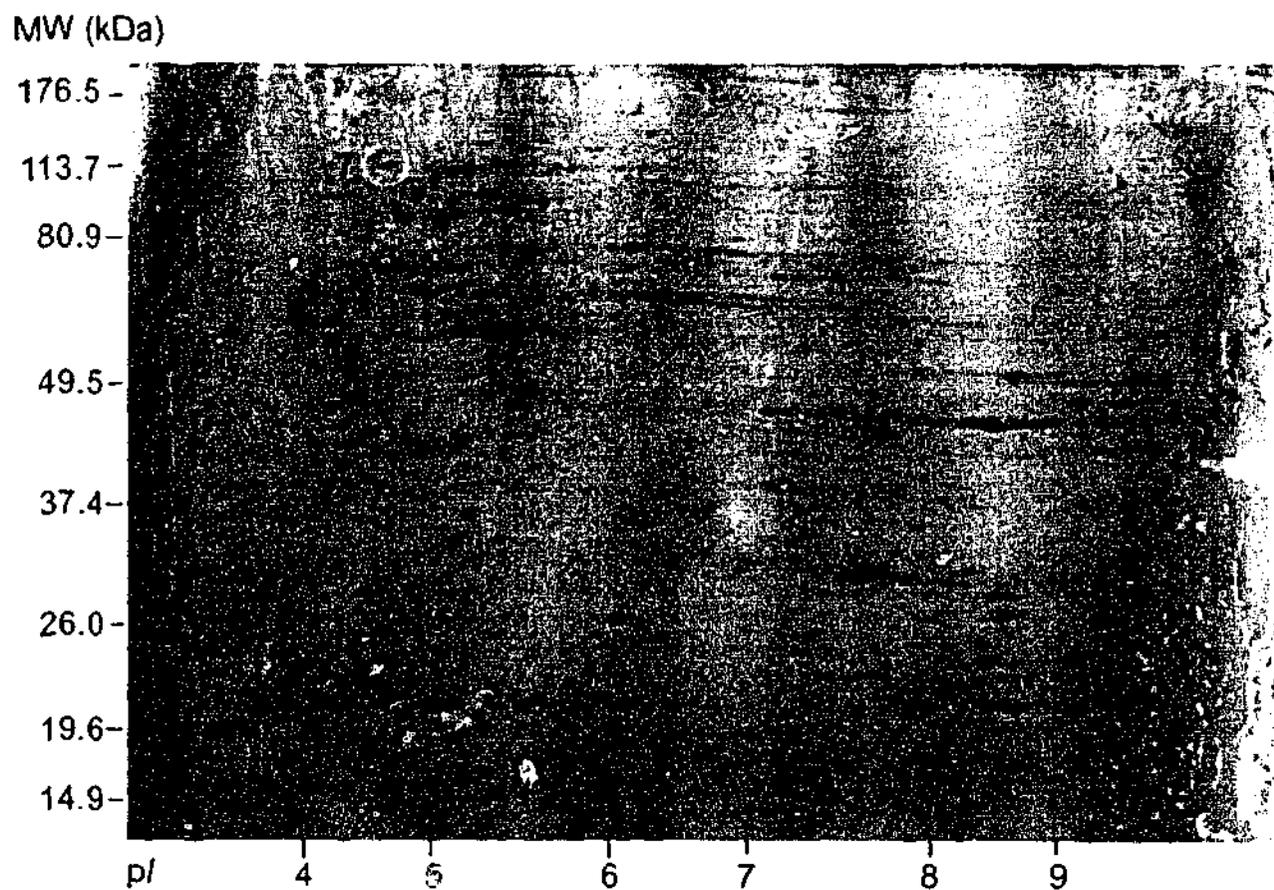
Figure 6-14

Two-dimensional gel separation of cellular proteins from human vascular SMCs treated with PDGF, genistein and the kinase inhibitor. Protein was isolated from human vascular SMCs treated with PDGF (50 ng/mL), genistein (100 µmol/L) and KI (1 µmol/L) for 24 h. Protein (500 µg) was separated on a pH 3-10 strip followed by molecular weight separation on a 12.5% separating gel and stained with Coomassie Blue at 60°C. The green circled protein spot was detected only in cells treated with PDGF/genistein/KI and was arbitrarily assigned a number (7).

Spot No	Protein Description	MW (kDa)	pI	% Protein covered by matched peptides
1	MHC Class 1 antigen	23.7	5.4	20.4%
3	Toll/interleukin-1 receptor domain-containing adapter protein OR	25.3	6.0	16%
	Ras-related protein Rab-6B	23.5	5.4	18%
5	Calcium/calmodulin-dependent protein kinase type II delta chain	56.3	7.0	44%

Table 6-1

Identification results of proteins differentially expressed between cells treated with PDGF/genistein and PDGF/genistein/KI. Spots of interest were excised from the gels presented in Figure 6-13 and 6-14 and the protein was analyzed by mass spectroscopy and potential identity was recognized using MS-Fit (http://prospector.ucsf.edu).

6.3 Summary of results for Chapter 6

The studies presented in this chapter aimed to evaluate the cell culture system used to study the effects of fibrates (PPAR-α ligands) by characterizing the effects of atherogenic growth factors and hormones and agents other than fibrates, on vascular proteoglycans. This also involved a search for GAG elongation enzymes sensitive to a kinase inhibitor. In these studies we found that:

- Human vascular SMCs treated with TGF-β1 (0.01-3 ng/mL) showed a parabolic concentration-response for proteoglycan synthesis while the treatment of vascular SMCs with either PDGF (1-300 ng/mL) or AII (1-300 nmol/L) gave a sigmoidal log-concentration response for proteoglycan synthesis;

- The combination of TGF-β1 with PDGF had an additive effect on proteoglycan synthesis and GAG length that exceeded the stimulation following treatment with either growth factor alone;

- Treatment of vascular SMCs with calcium channel antagonists, felodipine and verapamil and a calcium channel agonist, Bay K 8644, decreased [^{35}S]-sulfate incorporation into proteoglycans in the presence and absence of either TGF-β1 or PDGF;

- Treatment of vascular SMCs with felodipine, under basal conditions and in the presence of TGF-β1, reduced GAG length with no effect in the presence of PDGF. Treatment of vascular SMCs with verapamil or Bay K 8644 increased the electrophoretic mobility of the HS/CSPG in the presence of PDGF;

- Treatment of human vascular SMCs with sodium chlorate (0.5-5 mmol/L) showed a concentration-related reduction in 4-sulfation of GAGs with a consequential increase in unsulfated disaccharides;

- The treatment of vascular SMCs using "differential pharmacology" with a potent inhibitor of GAG elongation (KI) and a broad tyrosine kinase inhibitor (genistein) in the presence of PDGF showed that there were three protein spots differentially expressed. These proteins were matched to MHC Class 1 antigen, Toll/interleukin-1 receptor domain-containing adapter protein or Ras-related protein Rab-6B and calcium/calmodulin-dependent protein kinase type II delta chain.

6.4 Discussion of results for Chapter 6

The data in this chapter has shown the response of human vascular SMCs to growth factors, hormones and pharmacological agents, other than PPAR ligands, that are important in the development and treatment of atherosclerosis and its related complications. The study of growth factors has shown that the stimulation of vascular SMCs with TGF-β1 results in a parabolic concentration response curve of proteoglycan synthesis as previously demonstrated by others (Merrilees & Scott, 1990). The unusual response of vascular proteoglycan synthesis to high concentrations of TGF-β1 may reflect an induction of apoptosis, which has been previously described to occur in these cells with TGF-β1 (5 ng/mL) (Hishikawa et al., 1999). A potential relationship between apoptosis and proteoglycan synthesis is interesting and has recently been investigated in endothelial cells (Johnson et al., 2004) however, further investigation of our findings were beyond the scope of this thesis. Vascular SMCs treated with PDGF showed a classical log concentration-response relationship for the stimulation of proteoglycan synthesis. The effect of angiotensin II to stimulate proteoglycans synthesis also followed a log-concentration relationship however the response was very modest, possibly due to a lack of expression of AII receptors that occurs in multiply passaged vascular SMCs (Bernstein & Berk, 1993; Gunther et al., 1982). Our data also indicates that the stimulation of

GAG elongation by PDGF and TGF-β1 is additive and thus supports the idea of multiple, independent signalling pathways controlling GAG elongation.

The study of the pharmacological regulation of proteoglycan synthesis included a brief investigation of calcium channel antagonists (CCAs). CCAs have been shown to decrease the incidence of coronary artery disease by inhibition of SMC proliferation and foam cell formation (Mason *et al.*, 2003). The data provided here not only indicates that there may be novel "pleiotropic" actions of these agents but highlights that calcium may be an important factor in regulating GAG elongation. Calcium channel antagonists have been previously described to reduce proteoglycan synthesis in vascular SMCs (Vijayagopal & Subramaniam, 2001) and in other cell types (Fagnen *et al.*, 1999), with a related reduction in proteoglycan size. We have confirmed these findings in human vascular SMCs and shown that a calcium agonist does not induce a reverse effect such as an increase in proteoglycan size. The inhibition of proteoglycan synthesis and reduction in GAG length following SMC treatment with CCAs occurred under serine/threonine (TGF-β1) and tyrosine kinase type activation (PDGF), which suggests that the calcium channel antagonists inhibit proteoglycan synthesis downstream of the growth factor pathways and closer to the GAG elongation pathway.

We have shown that the FACE technique is sensitive for the detection of changes in the sulfation pattern of GAG. Human vascular SMCs with sodium chlorate show a reduction in 4-sulfation, with no effect on 6-sulfation due to an inhibition 4-sulfotransferase (Greve *et al.*, 1988). As a result of reduced 4-sulfation, unsulfated proteoglycans are secreted from the cell and this was observed as a concentration-related increase in the $\Delta di0S$ product. These data also demonstrate the sequence of events for the sulfation of GAGs in vascular SMCs. The treatment of vascular SMCs with sodium chlorate does not alter 6-sulfation of GAGs suggesting that 6-sulfation precedes 4-sulfation in the Golgi.

The treatment of vascular SMCs with genistein does not alter the GAG length of secreted proteoglycans and this is in contrast to the specific KI which is a potent inhibitor of GAG elongation (Little *et al.*, unpublished observations). The KI was used to search for proteins potentially involved in GAG elongation. We used a "differential pharmacology" approach by treating human vascular SMCs with PDGF/genistein and PDGF/genistein/KI. The separation of cellular proteins from the two treatments by 2-DE resulted in the identification of three proteins which were matched to MHC Class I antigen, Toll/interleukin-1 receptor domain-containing adapter protein or Ras-related protein Rab-6B and calcium/calmodulin-dependent protein kinase type II delta chain. MHC Class I antigen is involved in the immune response to viruses. Heparin and other heavily sulfated GAGs have been shown to inhibit the upregulation of MHC Class I in human umbilical endothelial cells stimulated with interferon-γ (Yard *et al.*, 1998). Toll/interleukin-1 receptor domain-containing adapter protein mediates inflammation and the innate immune response (Aderem & Ulevitch, 2000). Rab proteins are a large family of Ras-related GTPases which regulate intracellular vesicle transport of proteins from the endoplasmic reticulum to the *cis* Golgi (Tisdale *et al.*, 1992) however, their precise molecular mechanism is not established (Aderem & Ulevitch, 2000). The differential protein expression in human SMCs suggests that the Ras-related protein Rab-6B has a lower expression in cells treated with KI. It is speculated that if the Ras-related protein Rab-6B was involved in the transport of proteoglycan core proteins, a down-regulation of the Ras-related protein Rab-6B could slow down the transport of proteoglycan core protein from the ER to the Golgi and thus potentially reduce the time allocated for GAG elongation (Fellini *et al.*, 1984), resulting in secretion of proteoglycans that have shorter GAG chains. In SMCs, calcium/calmodulin-dependent protein kinase II (CaM kinase II) mediates cellular responses to increased intracellular $[Ca^{2+}]$ and is involved in cell

migration (Pauly *et al.*, 1995) and modulates smooth muscle myosin light chain kinase sensitivity to $[Ca^{2+}]$ (Tansey *et al.*, 1992). The CaM kinase II is also involved in regulating voltage-activated calcium channels (McCarron *et al.*, 1992), sarcoplasmic reticulum Ca^{2+}/ATPase activity (Grover *et al.*, 1996) and is an intermediate in the activation of mitogen activated kinase signalling cascade induced by norepinephrine (Muthalif *et al.*, 1996). It is speculated that if a reduction in CaM kinase II leads to an inhibition of GAG elongation, this protein may be inhibited by other pharmacological agents which inhibit GAG elongation such as the CCAs (e.g. felodipine). The potent GAG elongation inhibitor (KI) may exert its effects on proteoglycans by mediating the differential expression of these proteins. None of the identified proteins appear to have a direct relationship with proteoglycan GAG elongation however, these proteins could be involved in the signalling or regulatory pathway. The role of the identified proteins in the GAG elongation pathway would need to be investigated further.

Chapter 7: Discussion

Macrovascular disease is a major complication of type 2 diabetes. Current therapeutic interventions are directed at risk factors for diabetes and cardiovascular disease and most novel agents have demonstrated 'pleiotropic' and beneficial vascular effects that reduce myocardial infarction associated with atherosclerosis. For example, the use of oral hypoglycemic agents (e.g. rosiglitazone) for the treatment of hyperglycemia (Bakris, 2003), ACE inhibitors for the treatment of high blood pressure (UK Prospective Diabetes Study Group, 1998a), statins (e.g. simvastatin) for the treatment of hypercholesterolemia (Haffner, 2003; Libby & Aikawa, 2003; Sowers, 2003) and fibrates (e.g. gemfibrozil) for the treatment of hypertriglyceridemia and reduced HDL (Barter, 2001; Despres, 2001) all demonstrate a reduction in macrovascular complications which appears to supplement the relevant metabolic actions of the agents. Fibrates are used for the treatment of hypertriglyceridemia in patients with and without diabetes. The studies described in this thesis have addressed the question as to whether or not fibrates/PPAR-α ligands have direct vascular actions on vascular SMC proteoglycan synthesis, fine chemical structure and composition which could be a possible mechanism by which these agents contribute to a reduction in macrovascular disease.

The major findings of the work are that the treatment of vascular SMCs with fibrates/PPAR-α ligands leads to altered proteoglycan synthesis which resulted in a reduction in proteoglycan:LDL binding *in vitro* (Chapter 4, Section 4.2.2). The reduction in LDL binding to proteoglycans from fenofibrate treated vascular SMCs is attributed to a combination of biochemical changes including a reduction in GAG chain length (Chapter 3, Section 3.2.7-3.2.9) and an alteration in GAG composition (Chapter 3, Section 3.2.11). It was further demonstrated that fibrates do not cause a change in the sulfation pattern of vascular proteoglycans (Chapter 3, Section 3.2.10), which would be a further potential source of altered LDL binding.

An increase in proteoglycan synthesis and a change in the length, composition and sulfation pattern of the GAGs during atherogenesis not only enhance smooth muscle cell proliferation and migration (Ross, 1993; Wight, 1989) but also leads to altered proteoglycan biosynthesis and increased retention of atherogenic LDL in the vessel wall (Williams & Tabas, 1995, 1998). The retention of LDL by proteoglycans in the vessel wall, initiates a cascade of events including lipoprotein oxidation and an inflammatory response and therefore, represents an important therapeutic target for reducing the development of atherosclerosis (Staels, 2002). Atherogenic growth factors present in vascular lesions such as TGF-β1, increase GAG length (Schonherr et al., 1993; Schonherr et al., 1991) which enhances lipoprotein binding (Little et al., 2002). Analysis of GAG chain length was undertaken by SDS-PAGE and size exclusion chromatography and we confirmed that human vascular SMCs treated with TGF-β1 show reduced mobility on SDS-PAGE (Chapter 3, Section 3.2.7), greater hydrodynamic size by gel filtration (Chapter 3, Section 3.2.9) and these proteoglycans showed increased binding to LDL (Chapter 4, Section 4.2.2). Additionally this thesis provides evidence that the combination treatment of PDGF/insulin on human vascular SMCs results in the synthesis of proteoglycans that also have increased binding to LDL (Chapter 4, Section 4.2.2). It was further demonstrated that smooth muscle cells treated with both a serine-threonine receptor agonist (TGF-β1) and a tyrosine-kinase receptor agonist (PDGF) show an additive effect on GAG elongation (Chapter 6, Section 6.2.1.2), suggesting that the signalling pathways of these growth factors independently control GAG elongation. The specific focus of this thesis was the role of fibrate/PPAR-α ligand treatment in perturbing growth factor induced atherogenic changes in proteoglycan structure.

Fenofibrate treatment attenuated GAG chain elongation in the presence of both TGF-β1 and PDGF (Chapter 3, Section 3.2.7) suggesting the actions of these fibrate must be well

downstream of either signalling pathway, and closer to the GAG synthesizing mechanisms. A reduction in proteoglycan GAG length has been shown following the treatment of vascular SMCs with calcium channel antagonists (Vijayagopal & Subramaniam, 2001), glucosamine (Tannock *et al.*, 2002a) and gemfibrozil (Nigro *et al.*, 2002). The reduction in GAG length following vascular SMC treatment with either calcium channel antagonists or glucosamine is associated with a reduction in binding to LDL. We have extended the concept that reduced GAG length determines reduced LDL binding by showing that vascular SMCs treated with fibrates/PPAR-α ligands produce proteoglycans with reduced GAG length and this manifests as a reduction in binding to LDL. If these *in vitro* effects of fibrates/PPAR-α ligands on vascular SMC proteoglycans were to occur *in vivo*, this would represent an additional vascular mechanism by which these agents may contribute to a reduction in macrovascular disease as has been observed in atherosclerosis susceptible patients in clinical studies (Diabetes Atherosclerosis Intervention Study Investigators, 2001; Faergeman, 2000; Rubins *et al.*, 1999).

An unanswered aspect of this project is whether or not the action of fibrates/PPAR-α ligands to reduce GAG length is mediated through PPAR-α. In accord with the data of Staels and colleagues (Staels *et al.*, 1998b), the SMCs used in these studies express PPAR-α (Chapter 3, Section 3.2.2). We have previously shown that the fibrate/PPAR-α ligand, gemfibrozil, has similar actions on proteoglycan synthesis and GAG length (Nigro *et al.*, 2002). We extended these findings to show that fenofibrate induces similar structural changes in GAG length but we have now demonstrated that GAG composition is also altered. Thus, as two structurally distinct fibrates (See Figure 1-2) show similar effects, we can tentatively conclude that the fibrate parent chemical structure has a consistent effect, possibly a chemical class effect, on proteoglycans to inhibit GAG elongation. This conclusion is partly supported

by our findings with the potent PPAR-α ligand GW7647. Unlike fenofibrate, GW7647 treatment of vascular SMCs in the presence of atherogenic growth factors did not inhibit GAG elongation (Table 7-1). GW7647, a urea substituted thioisobutyric acid with 5,000 times higher potency for PPAR-α than fenofibrate (Brown *et al.*, 2001), is structurally different from fenofibrate/gemfibrozil suggesting that the fibrate chemical structure is required for the observed effects to inhibit proteoglycan GAG elongation. At this stage we can conclude that the observed effects with fenofibrate are most likely a fibrate class effect and probably but not definitely acting via PPAR-α (Table 7-1; Chapter 3, Section 3.2.14).

Whether agents which bind to nuclear receptors are actually exerting their effects through their respective receptor is very difficult to assess. Most of the informative work available at this time relates to PPAR-γ studies. The PPAR-γ ligand troglitazone, inhibits the proliferation of PPAR-γ^{-/-} cells leading to the conclusion that the inhibition is independent of PPAR-γ (Bruemmer *et al.*, 2003). However, it has recently been shown by molecular biological and pharmacological techniques, that ligand activation of PPAR-γ can inhibit vascular smooth muscle cell proliferation by cell cycle dependent mechanisms (de Dios *et al.*, 2003). Furthermore, this report noted that the vascular effects of several thiazolidinedione PPAR-γ ligands correlates more closely with their lipophilicity rather than their PPAR-γ receptor binding affinity (de Dios *et al.*, 2003). Thus the complex nature of signalling via heteromeric nuclear receptors, such as PPARs, suggests that the extent of involvement of the receptor may depend upon concentrations of the ligand and levels of expression of the receptor (Bishop-Bailey *et al.*, 2002).

The effect of PPAR-α ligands to reduce GAG length (Chapter 3, Section 3.2.7-3.2.9) may be the result of the inhibition of the chondroitin sulfate synthase enzymes (Kitagawa *et al.*, 2001; Yada *et al.*, 2003a; Yada *et al.*, 2003b) or the inhibition of chondroitin polymerizing

factor (Kitagawa *et al.*, 2003), responsible for the polymerization of CS GAG chains. At this stage there are no reports of factors that regulate the expression/activity of these enzymes in vascular SMCs nor have the promoter regions of these enzymes/factors been determined or characterized. The potential effects of PPAR-α ligands on vascular SMC proteoglycan GAG elongation is summarized in Table 7-1.

Treatment of vascular SMCs using differential pharmacology with a potent inhibitor of GAG elongation (KI) showed that three protein spots were differentially expressed (Chapter 6, Section 6.2.2.4). One of the identified proteins, Ras related protein Rab-6B, showed reduced expression in cells treated with KI (Chapter 6, Section 6.2.2.4). Ras related protein Rab-6B is involved in regulating the transport of proteins from the ER to the *cis* Golgi (Tisdale *et al.*, 1992). In rat chondrocytes, the movement of a newly synthesized core protein from the ER to the Golgi occurs slowly (60-100 min) compared to GAG elongation, packaging and secretion from the Golgi (10-15 min) (Fellini *et al.*, 1984). Assuming that there is a fixed time to synthesize a complete proteoglycan, inhibition of Ras related protein Rab-6B by KI could further slow down the movement of a proteoglycan core protein from the ER to the Golgi, thus reducing the time allocated for GAG elongation and secretion from the Golgi. A potential consequence of reducing the time for GAG elongation in the Golgi is the secretion of proteoglycans with reduced GAG chain length, the result observed following treatment of vascular SMCs with KI (Chapter 6, Section 6.2.2.4). These studies require confirmation that Ras related protein Rab-6B is associated with the transport of proteoglycan core proteins from the ER to the Golgi. Whether or not the reduction in GAG length following treatment of vascular SMCs with fenofibrate (Chapter 3, Section 3.2.7-3.2.9) is related to an inhibition of Ras related protein Rab-6B is another ponderable.

The assessment of proteoglycan core protein synthesis lead to the observation that

proteoglycan core protein contributes to an appreciable amount (≈6.5%) of the total protein biosynthesis in human vascular SMCs (Chapter 3, Section 3.2.3). The analysis of proteoglycan core protein expression showed that under basal conditions, fenofibrate treatment of vascular SMCs resulted in an increase in core protein synthesis (Chapter 3, Section 3.2.3). Under these conditions, proteoglycans from fenofibrate treated SMCs were smaller due to a reduction in GAG length (Chapter 3, Section 3.2.7). Thus, it appears that fenofibrate treatment in the absence of growth factors has two effects on proteoglycans; 1) an increase in proteoglycan core protein expression and 2) a reduction in GAG length. These two opposing actions possibly explain why the treatment of vascular SMCs with fenofibrate did not alter the incorporation of [^{35}S]-sulfate into proteoglycans under basal conditions. The effect of the fibrates/PPAR-α ligands to increase total core protein synthesis, are in contrast to reports of the effect of a fibrate/PPAR-α ligand (bezafibrate) to inhibit versican mRNA expression in hepatocytes (Table 7-1) (Olsson et al., 2001). A peroxisome proliferating response element (PPRE) is not present on the promoter region of versican from HepG2 cells, even though bezafibrate decreased versican mRNA expression (Olsson et al., 2001). This report did not address whether or not fibrates/PPAR-α ligands could reduce proteoglycan core protein synthesis by a PPRE-independent mechanism. PPAR activation indirectly inhibits the regulation of NF-κB and AP-1 controlled genes important in the inflammatory processes of atherosclerosis (Neve et al., 2000) (see Chapter 1, Figure 1-4). It is known that there are AP-1 and NF-κB sites in the promoter regions of perlecan (Iozzo, 1998), biglycan (Ungefroren & Krull, 1996), decorin (Iozzo, 1998) and osteoglycin/mimecan (Tasheva, 2002) core protein genes. It is possible that PPAR ligands could indirectly inhibit proteoglycan core protein synthesis via these sites (Table 7-1).

The characterization of GAGs by enzyme digestion showed that decorin from vascular

SMCs treated with fenofibrate contained a reduced proportion of dermatan sulfate (DS) than decorin isolated from untreated cells (Chapter 3, Section 3.2.11). Dermatan sulfate GAGs arise from the epimerization of the chondroitin (CS) chain by the uryonosl C5-epimerase in the *trans*-Golgi network. The effect of fenofibrate to reduce the DS component of GAGs on decorin may be due to an inhibition of the epimerase as this is the limiting factor in DS biosynthesis (Tiedemann *et al.*, 2001). The iduronic acid content and 4-sulfation features of dermatan sulfate have been reported to give structural stability to the PG-LDL complex (Cardoso & Mourao, 1994; Gigli *et al.*, 1993). The effect of fenofibrate to reduce epimerization may contribute to the reduced binding to LDL observed by GMSA. Factors that regulate the expression/activity of the C5-epimerase have yet to be identified and the promoter region has not yet been characterized. The possibility that fenofibrate and other PPAR-α ligands regulate C5-epimerase expression or activity in human vascular SMCs has not been explored however, targeting this enzyme represents a potential mechanism for the reduction in dermatan sulfate GAG and reduced binding to LDL (Table 7-1).

Heparan sulfate proteoglycans such as perlecan, inhibit SMC proliferation *in vitro* (Benitz *et al.*, 1990; Castellot *et al.*, 1987; Fritze *et al.*, 1985). Degradation of HSPGs by heparan sulfate lyases enhances the conversion of SMC phenotype from contractile to synthetic (Campbell *et al.*, 1992). Vascular SMCs treated with fenofibrate showed that there was a reduction in proteoglycans with HS GAGs compared to untreated cells (Chapter 3, Section 3.2.11). These data might suggest that fenofibrate treatment may enhance the proliferation of SMCs by reducing HSPGs however, this does not occur because fenofibrate (Munro *et al.*, 1994) and gemfibrozil (Nigro *et al.*, 2002) treatment both reduce the proliferation of vascular SMCs. It appears that direct cell cycle effects of fenofibrate predominate other effects on proteoglycans. Hence, the potential impact outcome of

fenofibrate induced reduction in HSPGs in vascular SMCs on vascular biology or pathology is presently unknown.

The keratan sulfate proteoglycan, osteoglycin/mimecan, is expressed in non-proliferating SMCs (Fernandez *et al.*, 2003; Shanahan *et al.*, 1997). Treatment of vascular SMCs with fenofibrate increased the KSPG component of the different proteoglycan bands as assessed by keratanase digestion and SDS-PAGE. Treatment with fenofibrate/gemfibrozil may increase the expression of osteoglycin and this may be associated with the actions of fenofibrate (or other fibrates) to inhibit SMC proliferation (Munro *et al.*, 1994; Nigro *et al.*, 2002). Additionally, the role of osteoglycin/mimecan in LDL binding has not been reported, however if this proteoglycan bound to LDL with lower affinity than CS/DS proteoglycans and this particular proteoglycan had been increased following treatment with fenofibrate, then this may represent another contributing factor to the observed reduction in LDL binding. The expression and regulation of KSPG by PPAR ligands in vascular SMCs requires further analysis.

Very few studies have investigated the pharmacological control of GAG sulfation in vascular SMCs or any other cell type (Greve *et al.*, 1988). The inorganic molecule, chlorate, is a potent regulator of sulfation (Chapter 6, Section 6.2.2.2) suggesting that regulation by therapeutic agents is potentially possible. Our data showed that the treatment of vascular SMCs with fenofibrate did not alter the sulfation pattern of GAGs, as assessed by FACE (Chapter 3, Section 3.2.10). These data suggest that the actions of fenofibrate were specific for GAG elongation processes and altering GAG composition and these changes account for the observed reduction in LDL binding.

	PPAR-α dependent PPRE dependent	PPAR-α dependent PPRE independent	PPAR-α independent PPRE independent
Regulation of <u>core protein</u>	No	Yes	Yes
Evidence from published data/our observations	No PPRE on versican core protein gene (Olsson *et al.*, 2001).	Presence of NF-κB and AP-1 sites on core protein genes And as for next column	↑ core protein synthesis following treatment of vascular SMCs with fibrates/PPAR-α ligands (Chapter 3, Section 3.2.3)
Regulation of <u>GAG length</u>	No	Either	
Evidence from published data/our observations	GW7647 treatment of vascular SMCs does not reduce GAG length (Chapter 3, Section 3.2.14)	Treatment of vascular SMCs with less potent PPAR-α ligands, i.e. fibrates, reduce GAG length (Nigro *et al.*, 2002) and (Chapter 3, Section 3.2.7-3.2.9)	
Regulation of <u>GAG composition</u>	All may be possible		
Evidence from published data/our observations	Fenofibrate treatment of vascular SMCs reduces dermatan sulfate composition of GAGs (Chapter 3, Section 3.2.11)		
Regulation of <u>GAG sulfation</u>	No	No	No
	No effect of fenofibrate treatment on sulfation pattern of GAGs as assessed by FACE (Chapter 3, Section 3.2.10)		

Table 7-1

Potential PPAR ligand regulation of proteoglycans synthesis in vascular SMCs. PPARs may regulate the gene expression of core proteins or enzymes involved in GAG elongation and composition, through different mechanisms.

The treatment of vascular SMCs with fenofibrate reduces [^{35}S]-sulfate incorporation into proteoglycans, over a 24-h treatment period. We have shown that the reduction in [^{35}S]-sulfate incorporation is associated with a reduction in GAG length. An alternative interpretation of this data could be that the treatment of vascular SMCs with fenofibrate, increases the degradation of proteoglycans. Proteoglycans *in vivo* have a half-life of a few days to several weeks (Hascall *et al.*, 1991). Proteoglycan catabolism occurs through the action of proteases which degrade the core protein and release GAG-peptide fragments (Hascall *et al.*, 1991). Macrophage lysosomes contain heparan sulfate glycosaminoglycan digestion enzymes such as heparinase (Campbell *et al.*, 1992) however, there is little evidence to suggest that GAG degrading enzymes are present in vascular SMCs. The interstitial matrix degradation enzyme, metalloproteinase-9, is expressed by vascular cells (Kanda *et al.*, 2000; Libby & Lee, 2000). In human monocytes, fenofibrate treatment decreases the expression and activity of metalloproteinase-9 (Shu *et al.*, 2000). It is unlikely that the reduction in sulfate incorporation into proteoglycans following treatment of SMCs with fenofibrate, would be due to increased degradation of proteoglycans however this could have been investigated using a "pulse-chase" experiment. This would involve pre-labelling SMC cultures with [^{35}S]-sulfate for a short period of time (24 h), followed by removal of the radiolabel by washing the cells with culture medium. The cells would be "chased" with non-radiolabelled culture medium containing 1 mmol/L sodium sulfate (Na_2SO_4) either with fenofibrate or no drug for 24 h. The secreted and cell-associated proteoglycans would be analyzed for sulfate incorporation at regular time intervals during the 24 h chase period to determine if there is a difference in the balance between synthesis and degradation of proteoglycans from fenofibrate treated SMCs.

To complement the analysis of glycosaminoglycan synthesis by radiosulfate incorporation, we used [^{3}H]-glucosamine to label vascular proteoglycans following treatment with

fenofibrate. The treatment of vascular SMCs with fenofibrate showed an anomalous increase in the incorporation of [^{3}H]-glucosamine into proteoglycans but a decrease in GAG length (Chapter 5, Section 5.2.1). To determine whether or not increased incorporation of [^{3}H]-glucosamine was a fibrate/PPAR-α ligand specific effect or a general effect of PPARs, we tested the PPAR-γ ligand, troglitazone. The treatment of vascular SMCs with troglitazone also increased the incorporation [^{3}H]-glucosamine into proteoglycans and was also associated with reduced GAG length. Unlike [^{35}S]-sulfate which is only incorporated into proteoglycans, [^{3}H]-glucosamine is incorporated into both proteoglycans and hyaluronan. The assay used to quantitate glycosaminoglycans may precipitate both proteoglycans and hyaluronan hence, an increase in [^{3}H]-glucosamine incorporation may represent an increase in hyaluronan synthesis. Using two independent assays, we found that the treatment of vascular SMCs with PPAR ligands (fenofibrate and troglitazone) did not increase hyaluronan production, indeed hyaluronan synthesis was reduced. We found that the PPAR ligands increase glycolysis, thereby increasing the specific activity of the hexosamines and this is reflected in the measurement of proteoglycan synthesis. Changes in the specific activity of radiolabelled glucosamine have not been reported in vascular SMCs however, this phenomenon has been studied in other cell types (Kim & Conrad, 1976; Salustri *et al.*, 1989; Yanagishita & Hascall, 1985). The mechanism by which troglitazone increases glucose utilization in vascular SMCs is most likely due to an increase in glucose uptake because troglitazone has been shown to increase the expression of the glucose transporter (GLUT)-1 in these cells (Kihara *et al.*, 1998; Yasunari *et al.*, 1997). Glucose metabolism in vascular SMCs is not fully understood and neither is the effect of relevant vasoactive agents however, it is well established that the hyperglycemia of diabetes is associated with an acceleration of the development of vascular disease. A multitude of studies have attempted to demonstrate different effects of glucose on

vascular SMCs but with variable success (Little *et al.*, 2003; Suzuki *et al.*, 2001). The studies in this thesis have shown the effects of PPAR ligands on human vascular SMC carbohydrate metabolism, results in apparently anomalous effects on proteoglycan synthesis. We have shown however, that the effects result from a perturbation of cellular metabolism resulting in a change in the specific activity of the intracellular hexosamine precursor pools. There is much to understand about glucose metabolism in vascular smooth muscle to assist in developing a link between hyperglycemia, and accelerated cardiovascular disease.

Data is emerging that strongly supports the role of PPARs (including PPAR-α) in inhibiting the inflammatory mechanisms of the vasculature in atherogenesis, a process which follows lipid accumulation (Ross, 1999). The mechanisms include repression of NF-κB regulated inflammatory genes (PPAR-α and -γ ligands) (Chawla *et al.*, 2001), decreasing C-reactive protein (PPAR-γ ligands) (Haffner *et al.*, 2002) and decreasing monocyte chemoattractant protein 1 (PPAR-δ ligands) (Lee *et al.*, 2003; Plutzky, 2003). Our data extends the vascular effects of PPAR-α ligands (fibrates) to an additional area of the initiation of atherosclerosis being a reduction in proteoglycan associated LDL retention (Williams & Tabas, 1995, 1998) (Figure 7-1). In addition to the favourable effects on lipid metabolism, PPAR ligands (fibrates) may have multiple but complementary effects in the vasculature by modifying proteoglycan synthesis and inflammatory responses which may reduce LDL binding and inflammation, respectively (Figure 7-1). If the effects of fibrate treatment on vascular proteoglycan synthesis observed *in vitro* were manifest *in vivo*, these changes would contribute to the reduction in macrovascular disease observed with administration of these agents (Figure 7-1) (Diabetes Atherosclerosis Intervention Study Investigators, 2001; Rubins *et al.*, 1999).

Increased hyaluronan deposition is observed in atherosclerotic lesions (Evanko *et al.*, 1998)

and in other inflammatory processes such as arthritis (Toole *et al.*, 2002). Mucosal SMCs, in inflammatory bowel disease, increase hyaluronan synthesis and this is associated with increased capture of monocytes via CD44 recognition sites on hyaluronan (de La Motte *et al.*, 1999). This work has shown that PPAR-α and -γ ligands, fenofibrate and troglitazone respectively, reduce hyaluronan synthesis in non-human vascular SMCs, suggesting that there may be another mechanism by which these nuclear receptor ligands contribute to a reduction in the inflammatory response associated with atherosclerosis (Ross, 1999). In rat mesangial cells, high glucose medium stimulates the formation of heteromeric hyaluronan "cables" which promote the capture of monocytes (Wang & Hascall, 2003). As an extension of this project, it would be of interest to look at the formation of hyaluronan "cables" and monocyte capture, by immunostaining of hyaluronan, following treatment of vascular SMCs with PPAR ligands as a novel and potential mechanism by which these agents reduce the inflammatory response associated with atherosclerosis. In human lung fibroblasts, pro-inflammatory cytokines, IL-1β and TNFα, increase hyaluronan synthase (HAS)2 expression but not HAS3 (Wilkinson *et al.*, 2004). The data presented in this thesis provide the foundation for studying the expression of the various HAS enzymes following treatment of vascular SMCs with the PPAR ligands.

In extending this project, we would address whether or not PPAR-α was involved in vascular proteoglycan synthesis using a molecular biological approach. That is, we would assess and compare proteoglycan synthesis in PPAR-$\alpha^{-/-}$ vascular SMCs to PPAR-α wild type SMCs. PPAR-$\alpha^{-/-}$ mice were not available to us during this project however, they have recently become available from The Jackson Laboratory, Bar Harbor, ME, USA (Lee *et al.*, 1995). The potential availability of these animals and thus, establishment of PPAR-$\alpha^{-/-}$ SMCs, would provide an interesting model to extend these studies on proteoglycan synthesis and

address unanswered questions. The immunohistochemical characterization of the extracellular matrix, including proteoglycans in coronary blood vessels from PPAR-α null mice has yet to be studied. This is another potential area of research that would assist in determining the role of PPARs in regulating proteoglycan synthesis. There may be differences in the expression of vascular proteoglycan core proteins in the vessels of PPAR-α$^{-/-}$ null mice (Tordjman *et al.*, 2001). Proteoglycans from the PPAR-α$^{-/-}$ null mouse model could be radiolabelled *in vivo*, extracted from the coronary arteries and assessed for size, sulfation pattern, GAG type and binding to LDL. That is the length of the chains could be assessed by SDS-PAGE and size exclusion chromatography, the charge by analytical DEAE and sulfation using FACE. Finally these proteoglycans could be used in the GMSA to determine if there is any change in the binding to LDL.

It is appropriate to consider the implication of our *in vitro* study on the contribution of such actions to the therapeutic utility and efficacy of fibrate therapy and hyperlipidemia. Treatment regimes for dyslipidemia in type 2 diabetes and atherosclerosis-susceptible subjects may include the combination therapy of a fibrate with a statin. A recent report has shown that the statin response element overlaps with the PPAR-α response element on the apolipoprotein A-I gene (Martin *et al.*, 2001). Whether proteoglycan core protein genes or GAG synthesizing enzymes contain statin response elements or PPRE remains to be studied. However, a recent study has shown that simvastatin and cerivastatin treatment of non-human vascular SMCs decreases [^{35}S]-sulfate incorporation into proteoglycans and this is associated with reduced LDL binding (Meyers *et al.*, 2003). Future experiments could be conducted by treating human vascular SMCs with both a statin and a fibrate to determine whether or not the fibrate effects on proteoglycan biosynthesis, structure and GAG composition are enhanced by the pre- or concomitant incubation with the statin.

In conclusion, these studies provide evidence that fenofibrate modifies the structure of proteoglycans produced by human vascular smooth muscle cells by reducing the length of the GAG chains resulting in reduced binding to human LDL. Fibrate therapy favorably modifies the proteoglycan binding properties of LDL in humans (Wiklund *et al.*, 1996) and thus fibrate treatment represents a "two pronged" attack (Figure 7-1) on reducing proteoglycan:LDL interactions by beneficial alterations in the biochemistry of both complex macromolecules (Nigro *et al.*, 2002; Wiklund *et al.*, 1996). Fibrates are primarily used to lower triglycerides and raise HDL in subjects with type 2 diabetes (Despres, 2001; Diabetes Atherosclerosis Intervention Study Investigators, 2001; Vakkilainen *et al.*, 2002). The studies in this thesis show direct vascular actions of fenofibrate which may complement the metabolic actions of fibrates in reducing cardiovascular disease. Our data would predict that this vascular mechanism may contribute to a favourable outcome for the FIELD trial and related studies and advance the "response to retention" hypothesis as a rational model of atherogenesis for developing therapies based on targeting the vessel wall.

Ligand ⬤
e.g. fenofibrate

PPAR-dep
PPRE-dep
PPAR-α activation

PPAR-dep
PPRE-indep

PPAR-indep
PPRE-indep

Indirect vascular actions

Lipids
↓Triglycerides
↑ HDL
Modify proteoglycan binding site on LDL

↓ Inflammatory response

Direct vascular actions

Modify SMCs
↓ proteoglycan synthesis and modify structure
↓ proliferation
↓ migration

LDL retention

Atherosclerosis

Treated vessel

Figure 7-1

Direct and indirect vascular actions of PPAR-α ligands which contribute to a reduction in lipoprotein binding in the vasculature. PPAR-α ligands such as fenofibrate represent a "two-pronged" attack on reducing LDL retention associated with atherogenesis; **direct** vascular actions on smooth muscle cell (SMC) migration, proliferation and proteoglycan biosynthesis and **indirect** vascular actions on lipid metabolism and an inhibitory effect on the inflammatory response to vascular injury. Whether or not the effects of PPAR-α ligands on proteoglycans are dependent (dep) or independent (indep) of the receptor (PPAR) and the response element (PPRE) is summarised in Table 7-1.

Appendix I

1 WAY ANOVA WITH UP TO 12 COLS AND 13 ROWS
Enter data and lambdas into blue area

	Con	Feno	Trog	HG Con	Feno	Trog	COL7	COL8	COL9
row1	51.55	52.09	101.14	27.74	38	42			
row2	49.67	56.60	129.98	30.52	36	35			
row3	38.10	49.83	66.24	27.74	33	38			
row4	47.79	57.62	74.53	29.87	27	31			
row5									
row6									
row7									
row8									
row9									
N	4	4	4	4	4	4	0	0	0
SUM	187.100199	216.1291151	371.892748	115.87322	133.949936	145.699591	0	0	0
AVERAGE	46.7750497	54.03227878	92.9731869	28.9663051	33.4874841	36.4248977	#DIV/0!	#DIV/0!	#DIV/0!
SAMPLE SD	5.98470748	3.692542242	28.8171045	1.44495964	4.95493498	4.77093643	#DIV/0!	#DIV/0!	#DIV/0!
SEM	2.99235374	1.846271121	14.4085523	0.72247982	2.47746749	2.38546822	#DIV/0!	#DIV/0!	#DIV/0!
ss/n	8751.62111	11677.9486	34576.0539	3356.6508	4485.64636	5307.09268	0	0	0
1/n =	0.25	0.25	0.25	0.25	0.25	0.25	0	0	0

analysis of variance table

sov	df	SS	MS	F	T	Probability	Significance	Delta	SEM
b/n cols	5	11054.62728	2210.92546	14.2751141		0.0000	***		
LG Con vs feno	1	105.3347464	105.334746	0.68010684	0.8246859	0.4203	NS	-7.2572	-8.800
LG Con vs Trog	1	4268.535755	4268.53575	27.5603298	5.24979331	0.0001	***	-46.1981	-8.800
LG Con vs HG Con	1	634.1603112	634.160311	4.0945346	2.02349564	0.0581	NS	17.8067	8.800
HG Con vs feno	1	40.84595792	40.8459579	0.26372699	0.51354356	0.6138	NS	-4.5192	-8.800
HG Con Vs trog	1	111.2015464	111.201546	0.71798656	0.84734088	0.4079	NS	-7.4566	-8.800
COMP 6	1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
COMP 7	1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
COMP 8	1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
COMP 9	1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
COMP 10	1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
COMP 11	1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
SUM	11	#DIV/0!							
RESIDUAL	18	2787.834689	154.879705						
TOTAL	23	13842.46197							

HARMONIC N=	4			SEM=	6.22253375	SED=	8.79999162	
						LSD 0.05	17.2479836	
						LSD 0.01	22.6687784	

TABLE OF LAMBDAS

	Con	Feno	Trog	HG Con	Feno	Trog	COL7	COL8	COL9
LG Con vs feno	1	-1	0	0	0	0	0	0	0
LG Con vs Trog	1	0	-1	0	0	0	0	0	0
LG Con vs HG Con	1	0	0	-1	0	0	0	0	0
HG Con vs feno	0	0	0	1	-1	0	0	0	0
HG Con Vs trog	0	0	0	1	0	-1	0	0	0

Table A1

Example of 1-way ANOVA table used for statistical analysis of data. Data are entered into

columns and Table of Lamdas is used make statistical comparisons.

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