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Dehydroepiandrosterone Action In The Cardiovascular System

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by

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I can do all things through Christ who Strengthens me

(Phil 4:13)

To my father Ibrahim, mother Lily, brother Mina

and wife Vivian

Thank you for all your love and support

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DECLARATION

This thesis contains no material which has been submitted or accepted for the award of any other degree or diploma in any university.

No portion of this thesis has been previously published or written by another person, except where due acknowledgement has been made.

This thesis is less than 100 000 words in length.

[redacted]

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ABBREVIATIONS

acu	Arbitrary Compliance Units
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
Apo	Apolipoprotein
AR	Androgen receptor
AEBSF	4-(2-aminoethyl)-benzenesulfonylfluoride
BMI	Body mass index
BSA	Bovine Serum Albumin
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CD 4+	Helper T cells
cGMP	Cyclic guanidine monophosphate
CHD	Coronary heart disease
CNS	Central nervous system
CVD	Cardiovascular disease
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECG	Electrocardiogram
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethyleneglycol-bis(2-aminoethyl-tetraacetic acid)
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ET_A	Endothelin receptor A

ET_B	Endothelin receptor B
ET-1	Endothelin-1
FAI	Free androgen index
FBS	Fetal bovine serum
FMD	Flow mediated dilation
G-6-PD	Glucose 6-phosphate dehydrogenase
GTN	Glyceryl trinitrate
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HSD	Hydroxysteroid dehydrogenase
IMA	Internal mammary artery
LDL	Low density lipoprotein
LDV	Laser Doppler velocimetry
MAP	Mean arterial pressure
MAP-kinase	Mitogen activated protein - kinase
MBP	Myelin Basic Protein
MCF7SH	Breast cancer cell line
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffer solution
PDGF	Platelet derived growth factor
PGI_2	Prostacyclin
PGH_2	Prostaglandin H2
PKI	Protein kinase inhibitor
RPM	Revolutions per minute
SAC	Systemic arterial compliance
SEM	Standard error of the mean
SLE	Systemic lupus erythmatosis
SNP	Sodium nitroprusside
TCA	Trichlor acetic acid
TXA_2	Thromboxane A2
VEGF	Vascular endothelial growth factor

SYNOPSIS

The adrenal steroid dehydroepiandrosterone (DHEA) and its sulphated pro-hormone DHEAS are the most abundant circulating adrenal steroids in young healthy adults. DHEA levels decrease with age and studies suggest that the steroid hormone may play a protective role in the pathogenesis and incidence of coronary artery disease. In addition, DHEA has been suggested to have beneficial effects on aging, immune function, body composition and depression.

As DHEA is a natural precursor to androgens and estrogens in the presence of the appropriate enzymes, it is not clear whether DHEA exerts its effects directly or via conversion to androgens and/or estrogens. We therefore investigated, *in vitro*, the effects of DHEA on the proliferation of vascular smooth muscle cells and aortic endothelial cells in culture with and without the estrogen receptor antagonist ICI 182,780 and the androgen receptor antagonist flutamide, and compared them to those of estradiol and testosterone. In addition, we also studied, *in vivo*, the effects of chronic administration of DHEA (100mg/day for 3 months) on indices of endothelium dependent nitric oxide (NO) mediated dilation and arterial elasticity 36 healthy postmenopausal women.

Methods: *In vitro*, human vascular smooth muscle (VSMC) and bovine aortic endothelial cells were grown to confluence in DMEM with 10% FCS. Following serum deprivation for 24 hrs, cells were treated with DHEA, estradiol or testosterone (1 - 100nM) for 16 h, in the presence or absence of the estrogen receptor antagonist ICI 182,780 (100nM, 8 h) or the androgen receptor antagonist flutamide (100nM, 8 h). Cell proliferation was then

stimulated with platelet derived growth factor (PDGF) BB (10ng/ml) or 2.5% FCS (48h) for smooth muscle cells and endothelial cells respectively, and cell numbers were counted. The affinity of DHEA for estrogen and androgen receptors and its specific binding in intact cells was also investigated. Mitogen-activated protein (MAP) kinase activity was determined by immunoprecipitation of the kinases ERK-1, JNK and P38 followed by specific substrate protein phosphorylation.

In vivo, three non-invasive techniques were employed to assess vascular function: laser Doppler velocimetry (LDV) with direct current iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP); flow mediated dilation (FMD) of the brachial artery during reactive hyperaemia by high frequency ultrasound; and, arterial distensibility measured by applanation tonometry and Doppler velocimetry.

Results: *In vitro,* binding studies and western blot analysis confirmed the presence of estrogen (ER) and androgen (AR) receptors in both VSMCs and endothelial cells. Competitive binding assays demonstrated that DHEA does not bind to either ER or AR. However, specific binding sites for DHEA were found to be present on both VSMC and endothelial cells.

DHEA and estradiol significantly inhibited PDGF-BB induced-increases in VSMC proliferation by 49±4 and 53±3% respectively, while testosterone accentuated this effect, 217±9%. While estradiol-induced inhibition of the PDGF effect was abolished by ICI 182,780 ($p<0.05$) and testosterone-induced accentuation was abolished by flutamide ($p<0.05$), neither receptor antagonist influenced the inhibitory effect of DHEA.

Furthermore, analysis of cellular MAP kinase activity demonstrated that incubation of the cells with PDGF-BB plus or minus various concentrations of DHEA resulted in significant inhibition of the ERK-1 kinase activity stimulated by the growth factor in a concentration-dependent manner. Although both JNK and P38 were present in the cultured VSMC by Western blotting, the activity of either kinase was altered in the presence or absence DHEA and PDGF-BB.

In addition, DHEA, estradiol and testosterone significantly accentuated FCS induced-increases in endothelial cell proliferation by 24±1.9, 17±1.5 and 28±2.3% ($p<0.05$) respectively. While estradiol and testosterone-induced increases in endothelial cell proliferation were abolished by ICI 182,780 and flutamide ($p<0.05$) respectively, neither receptor antagonist influenced the stimulatory effect of DHEA. Analysis of MAP kinase activity demonstrated that DHEA significantly stimulated FBS induced increases in ERK-1 kinase phosphorylation.

In vivo, DHEA administration significantly increased endothelium-dependent brachial artery dilation and subcutaneous vascular reactivity as measured by FMD and laser Doppler velocimetry with direct current iontophoresis, respectively ($p<0.05$). There was no effect on nitroglycerin-induced vasodilation of the brachial artery or subcutaneous microvasculature. Furthermore, arterial elasticity was significantly increased following DHEA administration with no effect on SBP, DBP or MAP. DHEA significantly reduced total cholesterol from 6.2 to 5.5 ($p<0.05$) compared to placebo (5.9 to 5.9). There were no significant effects of DHEA on HDL or LDL cholesterols or triglycerides.

Conclusions: The studies presented in this thesis have shown, by both *in vitro* and *in vivo* approaches, a potentially beneficial role for DHEA in the setting of cardiovascular physiology. These studies also extend our current understanding of the potential mechanisms by which DHEA may contribute to the lowering of cardiovascular disease. In addition, these findings may provide valuable insight regarding the suitability of DHEA administration in humans.

Chapter 1

Background and Rationale

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1.1 CHAPTER OVERVIEW

Cardiovascular disease (CVD) is the single leading cause of mortality in the Western World. For this reason, an understanding of its pathogenesis is of great importance. It appears that major contributing factors include functional and structural alterations in the blood vessel wall, leading to the activation of coagulation mechanisms, increased proliferation and migration of smooth muscle cells and compromised endothelial function. However, there are clear gender differences regarding the incidence and clinical manifestations of CVD, with men being at greater risk compared to women. Cardioprotection is especially afforded in premenopausal women but is rapidly negated following menopause where the incidence of CVD-related morbidity increases approximately 40-fold (Lamberts, 1997).

The delayed onset of CVD in women and the observed cardiovascular benefits of hormone replacement therapy suggest the possibility of beneficial effects of sex hormones, particularly estrogens, in the protection against CVD. Indeed, it has been well documented that estrogens beneficially alter plasma lipid metabolism. Moreover, there is an accumulation of data showing that estrogens exert beneficial effects by direct actions on vascular structure and function.

While many of the effects of estrogens on vascular function are well documented, those of androgens are largely unknown. However, the impact of androgens on cardiovascular health has received increasing attention during recent years with studies reporting conflicting findings. It has been proposed that the discordant results observed between

studies of androgen action may reflect gender-related differences, in that androgens exert beneficial effects in men but potentially deleterious effects in women (English, 1997; Nishino, 1998). However, no conclusive evidence has been presented to support this hypothesis.

Dehydroepiandrosterone (DHEA) and its sulphated pro-hormone DHEAS are quantitatively the most abundant circulating steroids in man and have been implicated in the protection against CVD. Although generally considered to be a weak androgen itself, DHEA is a precursor to the androgens androstenedione, testosterone and dihydrotestosterne, as well as to estrogens via enzymatic conversion. Therefore, studying its role in cardiovascular physiology is not entirely straightforward, as it is unclear whether DHEA exerts its effects directly or through conversion to other steroids. An added complexity exists in this regard because a putative receptor for DHEA has not yet been identified in vascular tissue.

A limitation in the current literature regarding the relationship between androgens, particularly DHEA, and CVD is that the majority of investigations to date have been epidemiological or animal studies rather than prospective, placebo-controlled interventional studies. In addition, the lack of in vitro findings further highlights the deficiency in the current knowledge with regards to the cellular mechanisms by which androgens might exert cardiovascular effects.

This chapter aims to provide a broad overview into the role of sex hormones in the setting of cardiovascular physiology, with particular attention to the vascular

endothelium, smooth muscle, and mechanical arterial properties, all of which are integral components contributing to the maintenance of vascular function. Additional consideration will be given to the various non-invasive techniques used in the assessment of endothelial function and arterial mechanical properties in the outlined studies. Following this, the focus of the chapter will shift to review the current knowledge in the major areas of DHEA research, with particular emphasis on the area of cardiovascular health. A number of contentious issues are highlighted with those relevant to the area of DHEA and cardiovascular physiology being addressed in the chapters to follow.

1.2 SEX STEROID METABOLISM AND ACTION

1.2.1 Synthesis and Metabolism of Sex Hormones

The major sites of steroid hormone synthesis are the adrenal cortex (primarily DHEA), ovaries and placenta (estrogens), and testes (androgens). Sex hormones, glucocorticoids, and mineralocorticoids are synthesized from cholesterol via a series of enzymatic conversions. The first step in this process is the synthesis of pregnenolone. Pregnenolone itself is hydroxylated to yield 17α-OH pregnenolone or is converted to progesterone by 3β-hydroxysteroid dehydrogenase isomerase complex, which also converts 17α-OH pregnenolone to 17α-OH progesterone and DHEA to androstenedione.

DHEA, which is secreted primarily as the sulfated derivative DHEAS, and androstenedione, are the principal androgenic steroids produced by the adrenal cortex. DHEA(S) secretion is modulated by adrenocorticotropic hormone (ACTH) (For a recent review see Burger, 2002). Figure 1.1 illustrates the adrenal steroidogenic pathways in the metabolism of DHEA to androgenic and estrogenic metabolites. Briefly, steroid sulfotransferase converts DHEA to DHEAS, and the reverse reaction is mediated by the enzyme steroid sulfatase. In addition to androstenedione, DHEA is converted to androstenediol via the actions of 17β-hydroxysteroid oxidoreductase. Both androstenediol and androstenedione are converted to testosterone via the actions of 3β-hydroxysteroid dehydrogenase isomerase and 17β-hydroxysteroid oxidoreductase respectively. Testosterone can be further metabolized to 17β-estradiol and estriol by the

enzyme aromatase, or to dihydrotestosterone by 5α-reductase. Aromatase is also responsible for the conversion of androstenedione to estrone.

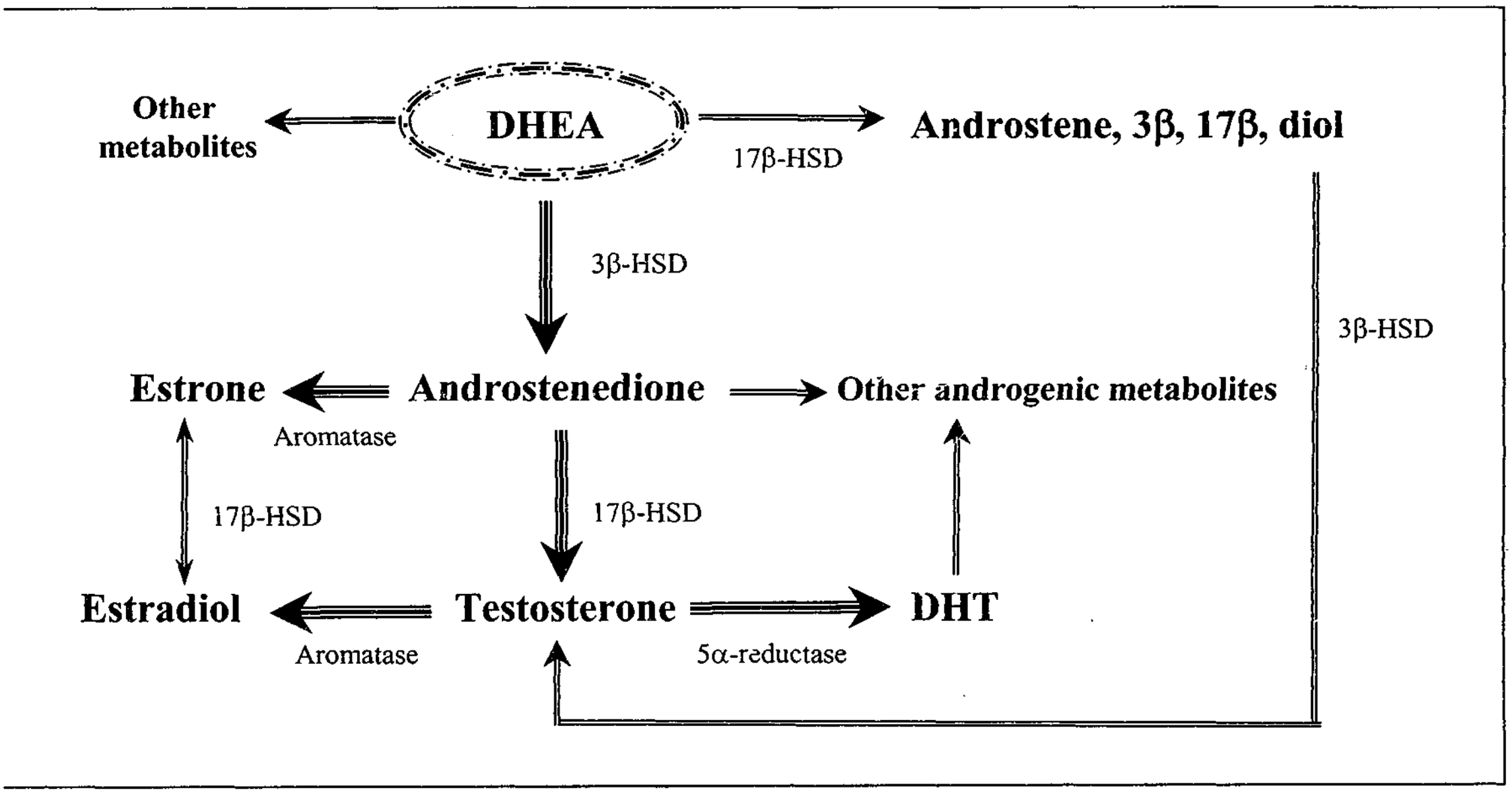

Figure 1.1
Schematic representation of the steroidogenic pathways involved in the conversion of DHEA to downstream androgenic and estrogenic metabolites

1.2.2 Production and Metabolism of Sex Hormones in Menopause and Male Ageing

A feature of the ageing human is that most physiological functions gradually deteriorate (Reviewed by (Lamberts , 1997)). There is increased incidence of coronary artery disease (CAD), impairment of central nervous system (CNS), reduced bone mineral density and muscle mass, and immune and vascular function. In addition, ageing is accompanied by marked changes in sex steroid production and secretion.

The most striking endocrine change in women is menopause, which takes place during the fifth or sixth decade of life. As menopause approaches, follicular activity gradually ceases and ovarian estrogen production and circulation diminish (Judd, 1982; Richardson, 1987; Richardson, 1990). The low levels of circulating estrogens seen in postmenopausal women arise from the peripheral vasculature and adipose tissue. In addition, the anterior pituitary gland secretes large amounts of gonadotropins, with circulating luteinizing hormone and follicle stimulating hormone levels rising above those seen in premenopausal years (Meldrum, 1981; Speroff, 1993; Speroff, 1993). It has long been accepted that the one of the main factors in determining the timing of menopause is the exhaustion of ovarian follicles. However, recent evidence suggests that the hypothalamic-pituitary unit of the brain also contributes to the onset of menopause (Hall, 2001).

Men experience less dramatic endocrine changes with ageing compared with women. Increased age in males is associated with a gradual decrease in circulating testosterone levels (Gray, 1991; Morley, 1997), which is brought about both by a decrease in Leydig

cell numbers and secretory capacity, and decreased gonadotropin secretion (Harman, 1980; Harman, 1982).

Circulating DHEAS levels in healthy adults are approximately 400 times greater than those of DHEA (Orentreich, 1984) and approximately 10 times greater than circulating cortisol levels (Ravaglia, 1996). DHEAS and DHEA levels are greatest during adolescence, peak in the third decade of life, and subsequently diminish gradually with age, reaching approximately 5% of peak levels by the age of ninety years (Orentreich, 1984; Ravaglia, 1996). DHEAS is produced at a rate of 3.5–20 mg/day during reproductive life and circulating concentrations vary between 1–4 μg/mL (Burger, 2002). The production rate of DHEA is 6-8 mg/day and circulating concentrations vary between 1-10 ng/mL (Burger, 2002). The decline in DHEA(S) levels occurs in response to a decrease in the number of functional zona reticularis cells in the adrenal cortex (Herbert, 1995; Hornsby, 1995; Labrie, 1995; Labrie, 1995). Circulating DHEA levels in women are higher compared to age-matched men (Zumoff, 1980; Zumoff, 1980; Sulcova, 1997). This is explained, in part, by ovarian production and regulation of DHEA metabolism, although it has been suggested that the ovaries account for less than 10% of DHEA production in women (Khorram, 1996).

1.2.3 Cardiovascular Physiology of Menopause and Male Ageing

The age-related decline in various physiological processes is more noticeable in women compared with men as they are often abruptly accelerated with the onset of menopause. The altered hormonal state seen in postmenopausal women is associated with a variety of physiological changes including vasomotor instability, depressed mood and altered

cognitive function, changes in body composition, loss of bone mass and increased incidence of cardiovascular disease (Christiansen, 1980; Ettinger, 1985; Riis, 1987; Drake, 2000; Duka, 2000; Verghese, 2000).

It is well established that the incidence and clinical manifestation of coronary artery disease is lower in women than in men at all ages. This "cardioprotection" is particularly evident in premenopausal women but declines after menopause. Many studies have suggested that estrogen-containing therapies in postmenopausal women reduce the risk of CAD to levels comparable to those of premenopausal women (Grady, 1992; Barrett-Connor, 1997; Barrett-Connor, 1997; Varas-Lorenzo, 2000). The putative cardioprotective effects of estrogens are attributable to a number of factors, including alterations in the lipid profile as well as direct effects on vessel wall physiology (Bush, 1987; Grady, 1992; Farhat, 1996; Farhat, 1996).

The view that estrogen affords cardioprotection in women, however, has recently been put into question with publication of the Heart Estrogen-Progestin Replacement Study (HERS) (Grady, 1998; Hulley, 1998). HERS was the first large-scale randomized, placebo-controlled study to investigate the effects of hormone containing therapies on clinical cardiovascular outcomes in postmenopausal women with established coronary artery disease (CAD). The study demonstrated that despite a significant reduction in plasma LDL and increases in HDL levels there was no beneficial effect of estrogen supplementation on CVD outcomes. Rather, it showed an increase in thromboembolic events and gall bladder disease in those women taking estrogen-containing therapy compared with those taking placebo.

While the effects of estrogens on cardiovascular function are well documented, relatively few studies have evaluated the impact of androgen supplementation on cardiovascular health. There have been no large-scale prospective studies conducted of the cardiovascular impact of treating humans with testosterone or dihydrotestosterone, and as a result, most of the current knowledge is based on physiological, epidemiological, and animal studies. These studies have yielded conflicting findings and it has been proposed that the discordant observations may reflect differential gender-specific effects, in that androgens exert beneficial effects in men but potentially deleterious effects in women (Alexandersen, 1996; English, 1997). However, to date, no conclusive evidence has been presented to support this hypothesis.

1.2.4 The Effects of Sex Hormones on Lipid Metabolism

1.2.4.1 Estrogens

Due to the cessation of ovarian production and secretion of estrogens and progesterone during menopause, the lipid profile of postmenopausal women is altered in a manner that is thought to make it more atherogenic (Matthews, 1989; Stevenson, 1993). However, it has been extensively shown that hormone supplementation counteracts this deterioration.

Estrogens have been shown to lower low-density lipoprotein (LDL) cholesterol and apolipoprotein (apo) B, and to increase high-density lipoprotein (HDL) cholesterol and apoA1 (Bush, 1987; Nabulsi, 1993; Samaan, 1995). The decrease in LDL as a result of estrogen administration has been attributed to the up-regulation of the LDL receptor

(Newnham, 1993). In addition, a study by Keaney et al. (1994) demonstrated that estrogens limit LDL oxidation in hypercholesterolemic pigs (Keaney, 1994). This supports other studies linking estrogens to decreased oxidation of LDL cholesterol and generation of atherogenic lesions (Wagner, 1991).

There has been conflicting evidence regarding the effects of the natural fluctuations of sex hormones during the menstrual cycle on plasma lipoproteins. Several studies have shown that there is little correlation between the varying hormones and lipoprotein metabolism (Williams, 2001). However, other studies have suggested a 10 – 12% decrease in total plasma cholesterol and LDL cholesterol concentrations during the luteal compared with the follicular phase (Kim, 1979).

1.2.4.2 Androgens

Androgenic effects on lipid metabolism have also been investigated in men and women and studies have demonstrated gender-specific actions (Khaw, 1991). Testosterone supplementation in hypogonadal, elderly men, has been shown to decrease total and LDL cholesterol levels with no significant alterations on HDL cholesterol (Zgliczynski, 1996). In women, the effects of adrogens on the lipid profile are less clear. A study by Goh et al. (1995) suggested that testosterone appears to promote an atherogenic lipid profile by significantly increasing triglyceride, total and LDL cholesterol levels while decreasing HDL cholsterol levels (Goh, 1995). However, other studies have reported that androgen supplementation in women not only reduces triglyceride, total and LDL cholesterol, but also reduces HDL cholesterol (Sarrel, 1998). Furthermore, a recent study investigating the effects of testosterone administration in postmenopausal women

already taking estrogen-containing therapy showed that testosterone opposes the beneficial effects of estrogen on the lipid profile (Penotti, 2001). The effects of DHEA on lipid metabolism are reviewed in section 1.7.1.6 (DHEA and cardiovascular health).

1.3 THE VASCULAR ENDOTHELIUM

The endothelium is a unicellular layer of tissue that forms the luminal surface of all blood vessels. Until recently, the functional role played by the endothelium in cardiovascular physiology was not appreciated, and it was thought only to act as a semipermeable membrane between the blood and the interstitium. However, it is now recognized to play a dynamic and essential role in regulating and maintaining blood vessel function.

1.3.1 The Endothelium in Cardiovascular Physiology

The vascular endothelium performs a number of roles in the circulatory system. These include contributions to the control of thrombolysis (Luscher, 1990), vascular growth (Ross, 1993) and secretion of cytokines, platelet and leukocyte interactions with the vascular wall (Luscher, 1990) and regulation of vasomotor tone (Moncada, 1977; Furchgott, 1980).

1.3.1.1 Cardiovascular Homeostasis

The endothelium is a dynamic functional organ with metabolic, secretory, synthetic and immunological functions, that are important in controlling cardiovascular homeostasis (Fishman, 1982). Under normal physiological conditions, the vascular endothelium provides a highly thromboresistant surface to flowing blood (Schafer, 1997), prevents leukocyte and platelet adhesion, and inhibits the proliferation of vascular smooth muscle cells. The endothelium also contributes to cardiovascular homeostasis by regulating the caliber of blood vessels depending on the changing hemodynamic and hormonal

environment. It does this by secreting vasoactive substances, which in turn alter vasomotor tone and maintain blood viscosity (Rubanyi, 1993). The endothelium's role in maintaining cardiovascular homeostasis may be compromised by certain physical or chemical factors that lead to endothelial cell injury or activation (Rubanyi, 1991).

1.3.1.2 Thrombosis

Thrombosis reflects an imbalance between procoagulant and anticoagulant mechanisms (Bombeli, 1997; Gross, 2000). As mentioned, under normal conditions the endothelium possesses thromboresistant properties important to the maintenance of cardiovascular homeostasis. The endothelium elaborates molecules that prevent fribrin accumulation, and antiplatelet mediators. In addition, reactions involving thrombin mainly occur at the endothelial interface (Machovich, 1986).

Two molecules that stimulate platelet activation, thrombin and adenosine diphosphate, also promote the release of prostacyclin (PGI_2) from the endothelium, thereby inhibiting platelet aggregation (Moncada, 1977). In response to noradrenaline, vasopressin and thrombin-induced coagulation the endothelium also secretes tissue plasminogen activator, a potent thrombolytic agent (Hekman, 1987). In addition, endothelium-derived nitric oxide (NO) has been implicated in the reduction of platelet aggregation and adhesion (Golino, 1992; Yao, 1995). NO has also been shown to reduce the activation of circulating platelets and platelet-rich thrombosis in pulmonary hypertension (Nong, 1997).

In pathophysiological states, the equilibrium of prothrombotic and antithrombotic mechanisms is disrupted and the endothelium is no longer capable of maintaining vascular homeostasis. Excessive endothelial cell activation leads to increased levels of circulating prothrombotic substances (Nawroth, 1986; Nawroth, 1986; Rodgers, 1988; Bombeli, 1997). Table 1.1- details the balance of prothrombotic and antithrombotic agents expressed by the vascular endothelium.

Prothrombotic	Antithrombotic
Von Willebrand factor	Thrombomodulin
Plasminogen activator inhibitor-1	Tissue-type plasminogen activator
Tissue factor	Tissue factor pathway inhibitor
Thrombin receptor	Heparan sulfate
Annexin V	Endothelial cell nitric oxide synthase
Vascular cell adhesion molecule-1	Prostacyclin
Intracellular adhesion molecule-1	
E-selectin	
P-selectin	

Table 1.1
Prothrombotic and antithrombotic agents expressed by the vascular endothelium. (Adapted from Gross 2000)

1.3.1.3 Vascular Tone

A similar complexity exists in the endothelium's role in regulating vasomotor tone. The endothelium synthesizes and secretes vasodilator substances such as NO, prostacyclin (PGI_2), and the endothelium-derived hyperpolarizing factor (EDHF). The endothelium also liberates vasoconstrictor substances like endothelin-1 (ET-1) and other vasoconstrictor eicasanoids.

Under normal physiological conditions, the calibre and tone of blood vessels is maintained in a relaxed state primarily by the constitutive production of endothelium derived NO (Griffith, 1987; Palmer, 1987; Griffith, 1989). However, in states of endothelial dysfunction, such as atherosclerosis, in which the bioactive NO are reduced, the relatively unopposed actions of ET-1 promote vasoconstriction and smooth muscle proliferation (Lopez, 1990).

1.3.1.3.1 Endothelium-Derived Vasodilator Substances

1.3.1.3.1.1 Nitric Oxide

Discovered in 1980 (Furchgott, 1980) as an endothelium-derived reflaxing factor (EDRF), the many biological functions of NO, a structurally simple heterodiatomic molecule, have only been recently appreciated. NO has been shown to reduce platelet aggregation and leukocyte attachment to the vessel wall (Azuma, 1986; Radomski, 1987; Radomski, 1987; Radomski, 1987), inhibit vascular smooth muscle cell (VSMC) proliferation and tone (Garg, 1989; Taguchi, 1993; Fukuo, 1995), and regulate lipoprotein metabolism (Rubbo, 2000; Kotamraju, 2001).

The similarities between EDRF and NO in stimulating soluble guanylate cyclase (Rapoport, 1983) and inactivating hemoglobin and superoxide anions (Martin, 1985) had led to the proposition that the two molecules may be identical. Subsequent studies on the relaxation of the rabbit aorta by sodium nitrate (Furchgott, 1992), the pharmacology of EDRF (Ignarro, 1988), and measurement of NO production in endothelial cells (Palmer, 1987) further strengthened this hypothesis.

In endothelial cells, NO production occurs via a two-step conversion process. The first is the conversion of L-arginine to the intermediate N-hydroxy-L-arginine by electron donation from NADPH. The second step involves the oxidation of N-hydroxy-L-arginine to produce L-citrulline and NO (Palmer, 1988; Stuehr, 1991). Both of these processes are catalysed by the enzyme nitric oxide synthase (NOS) which has three known isoforms; endothelial, inducible and neuron NOS. Endothelial NOS (eNOS) is constitutively expressed in endothelial cells and the brain, and has been mapped to chromosome 7 (Marsden, 1993). In addition, the activity of eNOS has been shown to be calcium and calmodulin dependent (Lopez-Jaramillo, 1990; Forstermann, 1991). Both in vitro and in vivo studies have shown that the upregulation of eNOS expression and NO production from the endothelium may contribute to the improvement of vascular function seen in exercise and hormone-regulated models (Sessa, 1994; Hayashi, 1995; Hayashi, 1997). It has also been suggested that eNOS gene expression is, at least in part, regulated by mechanical stimulus (shear stress) (Sessa, 1994).

Vacular endothelial cells synthesize NO from the terminal guanido nitrogen atoms of L-arginine (Palmer, 1988). Once synthesized, NO diffuses from the endothelial cells to

underlying smooth muscle cells. The vasodilator properties of NO are largely due to its interaction with the iron atom of the heme prosthetic group of guanylate cyclase, causing its activation and subsequent increase in intracellular cyclic guanidine monophosphate (cGMP). cGMP production promotes a calcium efflux from VSMC resulting in vasorelaxation (Izumi, 1995; Walsh, 1995). Figure 1.2 is a schematic representation illustrating the mechanism of NO-mediated vasodilation as outlined above.

1.3.1.3.1.2. Prostacyclin

Prostacyclin (PGI_2) was the first endothelial-derived vasodilatory compound to be identified in the late 1970s (Moncada, 1979). PGI_2 is an intracellular signaling molecule that is synthesized from arachidonic acid by the enzyme cyclo-oxygenase following the stimulation of endothelial cells. This has been demonstrated both in vitro and in vivo (Moncada, 1977) by a variety of factors including thrombin, transfer of prostaglandin endoperoxides from platelets, contact with activated leukocytes, or stretching of the arterial wall (Weksler, 1977; Vane, 1990).

A PGI_2-specific receptor (the IP receptor) is present on vascular smooth muscle as well as on platelets (Coleman, 1994), indicating that PGI_2 acts to modulate the functions of these two cell types. This is supported by studies showing that the main physiological actions of PGI_2 are the prevention of platelet aggregation (Moncada, 1976; Vane, 1990) as well as the modulation of underlying smooth muscle tone (Shepherd, 1991). PGI_2, however, is not constitutively produced in endothelial cells and thus does not appear to regulate basal systemic vascular tone (Lefroy, 1993).

In addition, PGI_2 has been shown to have anti-atherogenic properties. This is evidenced by studies showing the inhibition of mitogen release from platelets, vascular endothelium and macrophages, and the inhibition of cholesteryl ester accumulation in macrophages and their conversion into "foam cells" (Willis, 1986). PGI_2 has also been successfully used to treat clinical complications of peripheral vascular disease (Vane, 1995). Figure 1.2 illustrates the mechanism of PGI_2-mediated vasodilation.

1.3.1.3.1.3. Endothelium Derived Hyperpolarizing Factor

Studies showing that acetylcholine stimulates endothelial-dependent vasodilation as well as hyperpolarization of vascular smooth muscle cells have led to the suggestion of the existence of a vasodilatory substance distinct from NO and PGI2 (Komori, 1988; Chen, 1991; Nagao, 1991). This viewpoint is further supported by studies showing that the observed hyperpolarization and reduction in vascular tone is not affected by nitric oxide synthase inhibitors, or cyclooxygenase and lipoxygenase antagonists, but is abolished by calcium-activated potassium channel antagonists (Huang, 1988).

Although receiving considerable attention, the chemical nature and identity of the endothelium-derived hyperpolarizing factor (EDHF) remains obscure with no definite indication to its identity. While the contributions of EDHF in maintaining resting membrane potential in vascular smooth muscle have been noted (Beny, 1988; Thollon, 1999), it has been suggested that EDHF is a less reactive compound than NO (Plane, 1994) and does not exhibit characteristics similar to those of prostanoids (Feletou, 1988; Nagao, 1992). However, like constitutively produced NO, EDHF release from

endothelial cells appears to require increased intracellular calcium levels (Chen, 1990) (Figure 1.2).

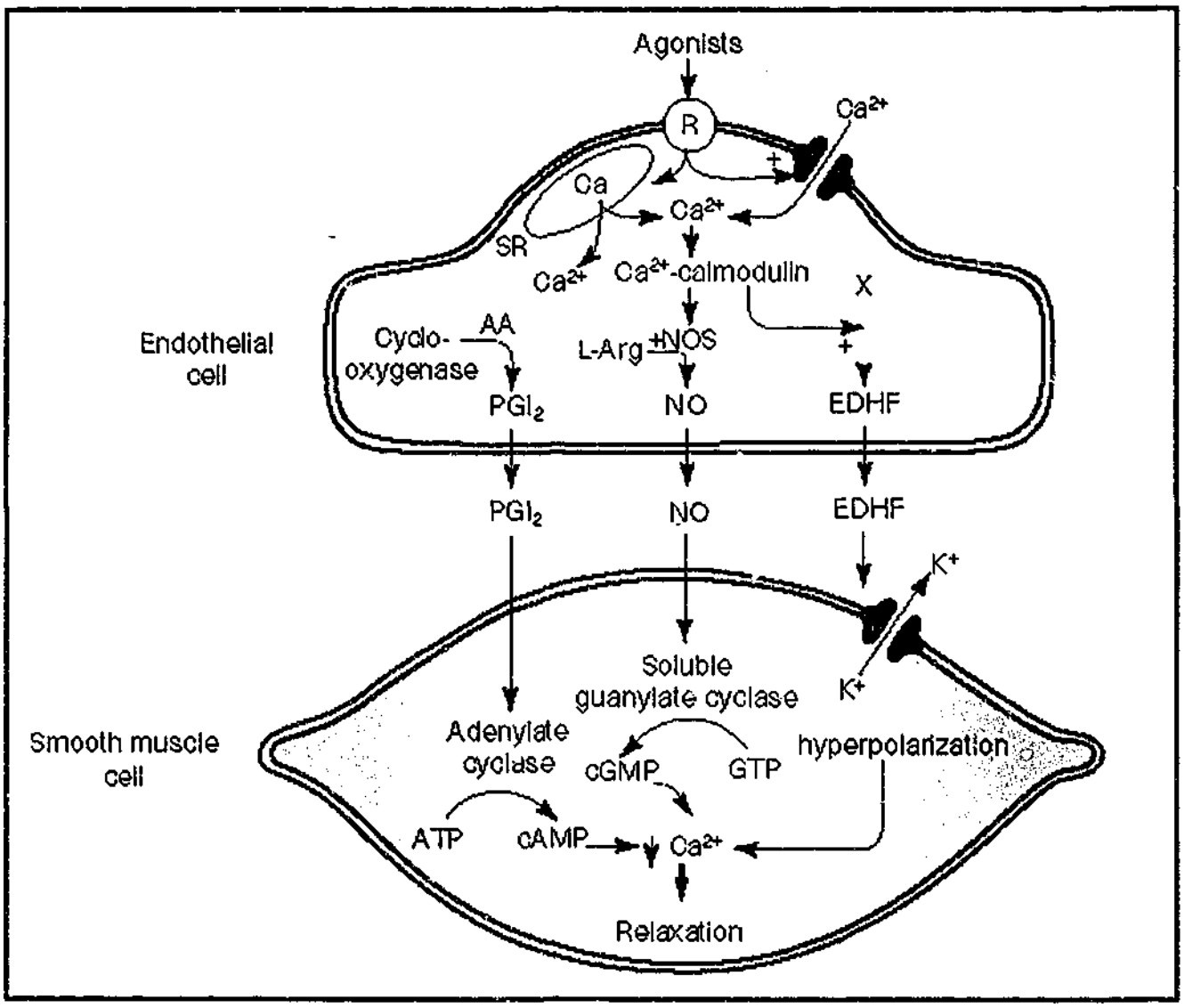

Figure 1.2
Schematic representation illustrating the mechanisms involved in nitric oxide, prostacyclin and endothelium-derived hyperpolarising factor endothelium-dependent vasodilation
(From Vanhoutte, 1998)

1.3.1.3.2 Endothelial derived Vasoconstrictor Substances

1.3.1.3.2.1 Endothelin-1

Endothelial cells also contribute to the maintenance of vascular tone by synthesizing and elaborating vasoconstrictor substances, the most potent of which is endothelin-1 (ET-1) (Yanagisawa, 1988; Yanagisawa, 1988; Levin, 1995). Endothelins make up a family of 21-amino acid peptides produced by many cell types. However, of the three isoforms of endothelins identified to date, only the ET-1 isoform is produced by endothelial cells. The other members of the family, endothelin-2 and endothelin-3, appear to have little effect on human cardiovascular function.

ET-1 is generated from a physiologically inactive 31 amino acid precursor, pre-endothelin-1 (Haynes, 1994; Xu, 1994), and through the action of endothelin converting enzyme (ECE) (Ikegawa, 1990; Pollock, 1991). ET-1 is released from endothelial cells in response to a number of stimuli, including thrombin, adrenalin, angiotensin II, hypoxia and shear stress. It exerts its actions by binding to endothelin specific receptors (ET_A or ET_B) (Hosoda, 1991; Ogawa, 1991). Both ET_A and ET_B receptors are expressed in vascular smooth muscle cells. Although both receptor subtypes are involved in mediating increased vascular tone (Moreland, 1992; Seo, 1994), ET_A receptors appear to be the main mediators of vasoconstriction (Davenport, 1994). ET_B receptors are found primarily on endothelial cells and have been shown to mediate the release of vasodilator substances like NO and PGI_2 (Gellai, 1997; Stephenson, 1997; Morawietz, 2000).

1.3.1.3.2.2 Vasoconstrictor Eicasanoids

Thromboxane A2 (TXA_2) and prostaglandin H2 (PGH_2) are constrictor factors also secreted by the endothelium (Haberl, 1987; Kanmura, 1987; Ito, 199[illegible]. They activate the thromboxane receptor in smooth muscle cells and platelets, in opposition to the effects of nitric oxide and prostacyclin. In contrast to ET-1, which has no direct platelet effects, TXA_2 and PGH_2 activate platelets, and as such, play an important role in thrombosis and platelet aggregation. Thromboxanes, together with the prostaglandins, make up the prostanoid subgroup of the eicosanoids.

TXA_2 and PGH_2 are lipid metabolites of arachidonic acid that are synthesized from activated platelets and vessel wall tissues. They have been described as potent vasoconstrictor agents for VSMCs acting through a common pharmacologically defined binding site, the TXA_2/PGH_2 receptor (Morinelli, 1990). Furthermore, TXA_2 has been shown to be a potent stimulator of VSMC proliferation, and therefore, may contribute to the pathogenesis of CAD (Ishimitsu, 1988; Morinelli, 1994; Sachinidis, 1995).

Thromboxanes are thought to be antagonists of PGI_2, a vasodilator eicasanoid (Minuz, 1988). An imbalance between the two in the favor of thromboxane will lead to initiation of platelet aggregation, acute inflammatory response and vasoconstriction (Narain, 1994). It has also been shown that TXA_2 may accelerate the progression of atherosclerotic CVD by inducing endothelial cell apoptosis and inhibiting endothelial cell migration and proliferation (Ashton, 1999; Gao, 2000).

1.3.1.4 Sex Hormones and Vascular Endothelial Function

Epidemiological studies have defined a sexually dimorphic pattern in the development of atherosclerotic vascular disease (reviewed in section 1.1). Consequently, the impact of sex hormones on vascular function has increasingly gained attention. There are a number of reviews summarizing the effects of sex hormones, particularly estrogens, on endothelial function and the cardiovascular system generally (Farhat, 1996; Barrett-Connor, 1997; Hutchison, 1997; Fisman, 1999; Fisman, 1999; Pines, 1999), the scope of which is beyond this thesis. It is therefore the aim of this section to provide a broad overview on current knowledge in the area of sex hormones and endothelial function.

1.3.1.4.1 Estrogens

Ageing has been associated with a progressive decline in endothelial function (Taddei, 1995). In addition, vascular resistance increases after menopause (Pines, 1991). Estrogen administration has been found to provide protection against the age-related decline in endothelium-dependent arterial reactivity in postmenopausal women (Sorensen, 1995; McCrohon, 1996; Gerhard, 1998). These studies have been supported by other investigations which have shown that estrogen supplementation in postmenopausal women increases peripheral blood flow and decreases impedance to blood flow (Gangar, 1993; Sudhir, 1996).

Arterial tone is determined, in large part, by the net influence of endothelium-mediated vasoconstrictor and vasodilatory pathways (Koller, 1994). Estrogens have been shown to potentiate endothelium-mediated vasorelaxation via direct effects on the production of vasodilator substances, particularly NO. In vitro studies have shown that estrogen

promotes the synthesis of eNOS (Wellman, 1996; Nuedling, 1999; Stefano, 2000). In addition, studies have also demonstrated increases in NO secretion following estrogen administration in vivo (Gilligan, 1994; Gilligan, 1994; Sudhir, 1996; Takahashi, 1997). These studies have been supported by others showing that endothelium-dependent vasodilatation is improved with estrogen administration (Gilligan, 1995). Animal studies further support the role of estrogen in maintaining endothelium-dependent vasodilation, evidenced by the observation that estrogen increases muscarinic receptors and prostacyclin production in endothelial cells (Wren, 1992; O'Sullivan, 2001). Estrogen has also been shown to enhance endothelial cell attachment and proliferation (Morales, 1995). It has also been reported that estrogen protects against vascular injury by stimulating endothelial cell migration and proliferation by mechanisms involving the up-regulation of plasma NO (White, 1997).

In addition to stimulating the production of vasodilator substances, estrogens also regulate vasomotor tone through influences on endothelium-derived vasoconstrictor substances. Estrogen has been shown to decrease ET-1 levels and block endothelin receptors, which subsequently reduces vasoconstriction (Sullivan, 1996). A recent study showed that estrogen replacement in ovariectomized rats suppresses PGH_2–induced vasconstriction (Davidge, 1998). Another study demonstrated that estrogen acts through a receptor-dependent mechanism to decrease PGH_2 synthase products from endothelial cells (Stewart, 1999). Estrogen has also been shown to decrease endothelial TXA_2 production (Sullivan, 1996). Other estrogenic effects on endothelial function, relevant to this thesis, are discussed in the appropriate chapters (Chapters 3 and 5).

1.3.1.4.2 Androgens

The impact of androgens on endothelial function has received considerably less attention than that of estrogens. There is, however, some evidence suggesting that androgens may exert effects on endothelial function, although results to date are conflicting. *In vitro* studies have demonstrated that testosterone and dihydrotestosterone may promote endothelial cell proliferation (Somjen, 1998). In addition, it has been shown that testosterone enhances endothelium-dependent vasodilation in coronary conductance and resistance arteries (Chou, 1996) and in men with coronary artery disease (Ong, 2000).

The optimism surrounding a potentially beneficial androgenic effect on endothelial function has, however, been put into doubt by other studies. It has been suggested that endothelial function in adult men is enhanced by androgen withdrawal (Herman, 1997), suggesting that androgens might suppress endothelial function. It has also been shown that testosterone diminishes bradykinin and calcium-induced vasorelaxation in porcine coronary arteries (Teoh, 2000). Likewise, Ceballos et al. (Ceballos, 1999) reported that testosterone increases vascular resistance and blocks adenosine-induced vasodilation. Additional studies have demonstrated that testosterone up-regulates TXA_2 receptor expression in VSMC (Higashiura, 1997) and enhances TXA_2 induced coronary artery vasoconstriction in guinea pigs (Schror, 1994). Studies investigating the effects of DHEA on vascular endothelial function are detailed in section 1.4.3 and in Chapters 3A and 5.

1.4 CLINICAL ASSESSMENT OF ENDOTHELIAL FUNCTION – GENERAL PRINCIPLES

The most widely used method for the evaluation of endothelial function *in vivo* has been the measurement of endothelium-dependent vasodilatory responses to pharmacological stimuli like acetylcholine, or mechanical stimuli like flow mediated dilation in conduction arteries and resistance vessels. In humans, several approaches have been employed to assess endothelium-dependent mechanisms of vasodilation, most of which measure vascular responses in regional circulatory beds, in particular the forearm or coronary circulation. The current review however, will detail the physiology pertaining to flow mediated dilation (FMD) in response to reactive hyperemia, and laser Doppler velocimetry (LDV) with direct current iontophoresis, the two non-invasive procedures used to assess endothelial function in the experiments reported in this thesis.

1.4.1 Flow Mediated Dilation

Endothelial cells not only respond to humoral factors but also to mechanical conditions created by blood flow and the cardiac cycle (Davies, 1995). Blood flow in the arterial circulation is pulsatile in nature. The vessel therefore, has a dual function: it is a conduit for blood flow as well as a compliant tube of changing cross-sectional shape and area with many side branches and bifurcations. Although the entire vessel wall, including the endothelium, smooth muscle and extracellular matrix, is subject to stretch as a consequence of pulsatile pressure, the laminar shear stress, exerted by the flow of blood, is received primarily by the endothelial surface (Davies, 1995).

An inherent characteristic of endothelial cells is that their alignment and morphology generally reflect the mean direction of shear stress within the vessel (Dewey, 1981; Levesque, 1986; Levesque, 1989). Both in vivo and in vitro studies have shown that endothelial cells align in the direction of blood flow (Flaherty, 1972; Goode, 1977; Nerem, 1981; Levesque, 1986; Levesque, 1989; Davies, 1995).

An important and rapid physiological adaptation to changes in arterial blood flow in conduit and resistance arteries is the acute regulation of arterial diameter. An increase in blood flow results in endothelial-dependent relaxation of smooth muscle cells and subsequent arterial dilation. In 1980, it was discovered that agonist-mediated vasodilation requires participation of the endothelium (Furchgott, 1980). Subsequent studies have confirmed the integral role of the endothelium in mediating arterial dilation in response to increased blood flow (Holtz, 1984; Rubanyi, 1986; Miller, 1988; Rubanyi, 1990).

The mechanism of shear stress-induced arterial dilation primarily involves the release of vasoactive factors from the endothelium, the principal component of which is NO and other closely related nitroderivatives (Palmer, 1987; Palmer, 1988; Moncada, 1991). Furthermore, prostacyclin has also been shown to be involved in shear stress induced dilation (Frangos, 1985; Grabowski, 1985).

The relative contributions of NO and prostacyclin in mediating flow-induced dilation appear to vary between vascular beds and pathophysiological conditions. For example, in arterioles of normotensive rat muscle it appears that both NO and prostacyclin are involved. However, in hypertensive rat models the NO-mediated dilation is impaired,

whereas the prostacyclin pathway is unaffected (Koller, 1994). In the basilar and brachial arteries and coronary microcirculation NO-mediated dilation appears to predominate (Kuo, 1992; Hashimoto, 1995).

Dilation of conducting arteries in response to increased blood flow has been used as a measure of endothelial function. One of the stimuli most commonly used to increase blood flow is reactive hyperemia caused by ischemia induced by temporary interruption of arterial blood flow, causing metabolic vasodilatation of the microcirculation and arterioles. Under the stimulus of ischaemia, anaerobic metabolism leads to the production of hydrogen ions (H^+), lactic acid, release of potassium (K^+), and other substances that are also capable of evoking vasodilatation.

A study by Meredith et al. (Meredith, 1996) demonstrated that endothelium-derived NO plays a major role in both reactive hyperemia and the maintenance of the hyperemic response following ischemia in the forearm. Another study involving chronic heart failure patients showed that physical training restores flow-dependent dilatation induced by reactive hyperaemia, and this was most likely attributed to enhanced endothelial release of NO (Hornig, 1996). The use of the response to an increase in blood flow as an index of endothelial function is validated by the experimental demonstration that flow-dependent vasodilatation of conductance arteries is determined by the release of nitric oxide from the endothelium (Holtz, 1984; Rubanyi, 1986; Cooke, 1990).

1.4.2 Laser Doppler Velocimetry with Direct Current Iontophoresis

In the peripheral circulation, endothelial function can be assessed in a noninvasive manner from the vasomotor response of the brachial artery or the forearm microcirculation, using ultrasound or plethysmography. Another means of assessing endothelial function and responses of the peripheral microcirculation non-invasively is by measuring blood flow by Doppler velocimetry coupled with direct current iontophoresis.

Iontophoresis refers to the transdermal migration of ions in solution following application of an electrical current (Harris, 1967). When a direct electric current is applied, the drug is repelled from the electrode of the same charge and subsequently penetrates the skin, whereas ions of the opposite polarity to the electrode are not transferred (Sloan , 1986). Laser Doppler velocimetry (LDV) is based on the "Doppler principle", which reflects a frequency shift of low-power laser light caused by moving red blood cells in subcutaneous arterioles and is used as an index of blood flow (Saumet, 1988).

This technique has been used to assess endothelial function by iontophoresing vasoactive substances known to act via the endothelium and measuring the subsequent changes in blood flux over a period of time (Komesaroff, 1998; Komesaroff, 1999). The extent of drug absorption into the skin is directly proportional to the magnitude and duration of the current applied (Sanderson, 1987; Phipps, 1989). Furthermore, the speed with which a drug can be delivered is influenced by its molecular weight (Chien, 1990) and the degree of ionization of the drug (Chantraine, 1986). However, the absolute dose

of drugs administered and the final concentrations achieved by iontophoresis remain difficult to ascertain. This is because many factors can contribute, including degradation of the drugs within the iontophoretic chambers, the extent of cutaneous penetration following electric current application, the washout rate of the drug in the microvasculature, and the variability of blood flow at basal conditions and in response to the administration of endothelium-dependent vasodilators (Pikal, 1990; Pikal, 1990; Pikal, 1990).

Investigations of vascular endothelial function using this or similar procedures have traditionally utilized acetylcholine (ACh) to assess endothelium-dependent vasodilation and sodium nitroprusside (SNP) for endothelium-independent vasodilation (Komesaroff, 1998; Komesaroff, 1999). ACh mediates endothelium-dependent vasodilation via interactions with nicotinic receptors present on endothelial cells. This results in NO release from the endothelium and subsequent vasodilatation. SNP is a nitric oxide donor which induces vasodilation by acting directly on the smooth muscle cells.

1.4.3 Summary and Clinical Implications of Endothelial Dysfunction

In summary, the endothelium plays a dynamic and essential role in regulating and maintaining blood vessel function by its elaboration of a diverse array of paracrine substances. Under normal physiological conditions, the functions of the endothelium that predominate are those promoting vasodilation, and inhibiting thrombosis and cellular proliferation. However, under certain adverse conditions, the balance of endothelial function shifts to favor vasoconstriction, thrombosis and cellular

proliferation, and these alterations in endothelial function participate in the pathogenesis of CVD, including hypertension and atherosclerosis (Clarkson 1997, Celermajer 1996, Celermajer 1994).

Endothelial function is regulated by humoral and mechanical factors. In addition, there are age-related modifications in the functioning of the vascular endothelium resulting in endothelial damage and dysfunction. Sex hormones, particularly estrogens, have been shown to improve endothelial function through effects on NO production and cell proliferation. The impact of androgens, particularly testosterone, on endothelial function has not been extensively investigated. Although current evidence suggests the possibility of beneficial androgenic effects, further investigation is warranted to elucidate the mechanisms by which androgens may affect endothelial function.

It has been suggested that DHEA may exert direct atheroprotective effects (see 1.7.1.6). However, whether any of these effects are mediated via influences on the vascular endothelium is yet to be determined. In addition, any observation regarding DHEA-mediated effects should be cautiously interpreted and consideration should be given to the mode of DHEA action in any particular setting. The reasons for this are highlighted by the absence of evidence supporting the presence of a DHEA-specific receptor in vascular tissue, and the fact that DHEA is metabolized into androgens and estrogens, which are known to influence many of the cardiovascular parameters assessed.

1.5 VASCULAR SMOOTH MUSCLE

Smooth muscle cells are the principal cell type found in the media of arteries and veins. Smooth muscle cells of the vascular system (VSMC) have long been recognized to posses a number of features important in the maintenance of cardiovascular health. These include their capacity to contract, proliferate, maintain vascular tone and synthesize proteins involved in maintaining connective tissue and extracellular matrix integrity. VSMC in the outer media of the vessel wall are oriented circumferentially, but appear more randomly distributed in the layers closest to the lumen (Kim, 1993; Slomp, 1997).

VSMC exhibit different morphological appearances according to their functional state. The VSMC phenotypes identified fall into two categories, namely epithelioid (synthetic) and spindle-shaped (contractile) cells (Ehler, 1995; Bochaton-Piallat, 1996). In the contractile state, VSMC contain abundant myofibrils and are surrounded by basement membrane. It is in this state that VSMC tone is regulated by a balance between the cellular signaling pathways that mediate the generation of force (contraction) and the release of force (relaxation). The signaling events that activate contraction include Ca^{2+}-dependent myosin light chain phosphorylation (Woodrum, 2001). However, when stimulated, contractile VSMC decrease the myofibril content and increase rough endoplasmic reticulum and Golgi bodies, thus transforming into the synthetic state. The synthetic phenotype is also referred to as a non-muscle or undifferentiated phenotype because it lacks the contractile differentiation markers SM22, calponin, h-caldesmon and smooth muscle myosin (Duband, 1993; Frid, 1994; Bergwerff, 1996). The remainder of

this review will focus on VSMC proliferation in arterial wall remodeling and atherosclerosis.

1.5.1 Vascular Smooth Muscle Cell Proliferation

Intimal VSMC migration, proliferation and synthesis of extracellular matrix proteins, leading to intimal thickening, in response to injury of several cell types are important early features in atherogenic CVD. During the initial stages of atherosclerotic lesion formation, VSMC migrate from the media to the intima and proliferate, giving rise to neointimal formation and increased extracellular matrix formation, resulting in the narrowing of the blood vessel (Newby, 1993; Newby, 1994; George, 1998). The coordinated regulation of cell proliferation and cell death therefore contributes to arterial remodeling (Xu, 2001). This realization has led to an increase in investigations into the factors responsible for VSMC proliferation.

In vitro cell cultures of VSMC demonstrate that serum provides all of the factors necessary for cell proliferation. Numerous studies have shown that the primary mitogenic component present in serum responsible for VSMC proliferation is a growth factor derived from platelets (PDGF) (Ross, 1974; Heldin, 1977; Heldin, 1979). Coupled with evidence to suggest that platelet accumulation occurs at sites of endothelial injury (Harker, 1976; Harker, 1978), this finding led to the hypothesis that platelets may play a significant role in the regulation of VSMC proliferation.

Indeed, subsequent studies demonstrated a correlation between the extent of endothelial injury, increased platelet utilization, and the formation of proliferative smooth muscle

atherosclerotic lesions at the sites of endothelial injury (Harker, 1976). Furthermore, in other models of endothelial injury, it was shown that platelet inhibition resulted in significant decreases in VSMC proliferation at the site of injury, whereas control models showed significant VSMC proliferative lesions (Moore, 1976; Friedman, 1977). In addition to endothelial integrity and PDGF, there is now increasing evidence that VSMC proliferation is regulated, at least in part, by a variety of other paracrine factors. VSMC have been shown to secrete growth regulatory substances that can stimulate the proliferation of muscle cells themselves (Ross, 1992).

Atherosclerosis is associated with a reduction in the capacity of the endothelium to promote vasodilation, which has been ascribed to a reduction in NO production (Bult, 1996; Vanhoutte, 1997) and an increase in vasoconstrictor substances (Goodwin, 2001). Studies have shown that NO inhibits VSMC proliferation (Garg, 1989; Kariya, 1989) and migration (Dubey, 1995), whereas ET-1 and angiotensin II have been shown to stimulate VSMC proliferation and migration (Taubman, 1989; Dubey, 1995) a potentially deleterious effect in the setting of atherosclerotic disease.

1.5.2 Sex Hormones and Vascular Smooth Muscle

The postulated protective role of the female sex hormones, in particular the estrogens, is substantially supported by studies showing a reduction in CVD in women receiving hormone containing therapies (see Section 1.2.3). The cardioprotective effects of estrogens have been largely attributed to their effects on the lipid profile. However, recent studies have shown that estrogen exerts direct effects on hemostatic processes and blood vessel wall physiology. Indeed, studies have shown that exogenous estrogen

supplementation inhibits the development of atherosclerotic coronary lesions, in part by inhibiting the growth and migration of VSMC (Rhee, 1977; Foegh, 1987; Wren, 1992).

In vitro studies have shown that estrogens inhibit VSMC proliferation (Fischer-Dzoga, 1983; Vargas, 1993; Yoon, 2001) by receptor dependent (Cathapermal, 1998; Lavigne, 1999) and independent mechanisms (Dubey, 2000; Karas, 2001). These reports have been supported by in vivo studies investigating the effects of estrogens on VSMC proliferation and migration in animal models of vascular injury. Estrogen has been shown to inhibit VSMC proliferation following balloon injury of the common and external iliac arteries of rabbits by inhibiting neointimal formation (VSMC proliferation and extracellular matrix formation) (Foegh, 1994). Another study has shown that estrogen inhibits balloon injury-induced myointimal VSMC proliferation by ~60% in Spague-Dawley rats (Chen, 1996).

Unlike those of estrogens, the effects of androgens on VSMC proliferation have received less attention. Two recent in vitro studies have demonstrated that testosterone and dihydrotestosterone accentuate the proliferation of cultured VSMC (Fujimoto, 1994; Somjen, 1998), an effect opposite to that of estrogen. Furthermore, while it has been shown in vivo that estrogen attenuates myointimal VSMC proliferation, the same study showed that testosterone did not affect VSMC proliferation in response to balloon injury (Chen, 1996). Studies investigating the effects of DHEA on VSMC behavior are detailed in Chapter 2A.

1.6 MECHANICAL PROPERTIES OF THE ARTERIAL VASCULATURE

The arterial system has been described as having two distinct functions: (i) a conduit function by means of which an adequate supply of blood is delivered to body tissues; and (ii) a cushioning function whereby the pulsatations resulting from intermittent ventricular ejection are dampened (Safar, 1996; Safar, 1996).

The arterial system is composed of an elaborate network of elastic vessels delivering blood from the heart to peripheral organs and tissues. Acting as elastic buffering chambers, the aorta and some of the proximal arteries store approximately 50% of the left ventricular stroke volume during systole. The cardiac cycle consisting of systole and diastole is associated with pressure changes. A typical pressure wave at the root of the aorta is shown in Figure 4.2. The height of the pulse is the systolic pressure (Ps) and the lowest point is the diastolic pressure (Pd) (Figure 4.2).

The elastic properties of the aorta drive this volume of blood into the periphery during ventricular diastole, thus ensuring a nearly even outflow of blood from the aorta into the circulation during the cardiac cycle even though the actions of the heart are pulsatile (Safar, 1996). The properties of large arteries have been likened to those of the Windkessel by Stephen Hales in the eighteenth century. The Windkessel was a storage tank used for the purpose of fire fighting and applied both conduit and cushioning functions to provide a continuous outflow of water when water inflow to the tank was of a pulsatile nature. The air-filled dome of the Windkessel acted as a cushion (in much the same way as the aorta) and the fire hose acted as the conduit (O'Rourke, 1992).

Arterial distensibility and compliance are mechanical properties of arteries and describe the elastic nature of the vessels. Therefore, the adequacy of the cushioning properties of the arteries depends on the compliance or ease with the arterial walls distend and contract.

In the event of the aorta stiffening, the "Windkessel" function deteriorates, causing a cascade of events which ultimately lead to detrimental consequences in terms of coronary blood demand and supply. Essentially, systolic blood pressure is elevated and diastolic blood pressure is reduced and so, pulse pressure is increased. Increased systolic blood pressure results in an increased cardiac afterload, and a decreased diastolic blood pressure results in decreased coronary perfusion which further stresses the cardiac demand and supply imbalance (Safar, 1995).

1.6.1 Arterial Compliance

Arterial compliance refers to the ease with which arteries can be distended. It is defined as the change in volume of a given vessel associated with the change in arterial pressure within that vessel (Equation 1.1). In a localized arterial segment it is a measure of the quantity of blood that can be stored for each millimeter of mercury pressure rise (O'Rourke, 1992).

Equation 1.1

Compliance = dV/dP

V = volume P = pressure

1.6.1.1 Factors Influencing Arterial Compliance

The elastic properties of the aorta have been shown to change significantly with certain pathological diseases as well as aging (Merillon, 1978). A study by Laogun and Gosling (Laogun, 1982) demonstrated that there is an increase in aortic elasticity during the first decade of life, and a subsequent gradual decrease as we age.

It has been demonstrated that advancing age results in the aorta and large arteries becoming progressively stiffer and more dilated (Avolio, 1985; Dart, 1991). Hypertension is thought to accelerate these changes (Safar, 1995). The precise mechanisms whereby arterial distensability is compromised with age are unclear. However, it has been hypothesized that over many decades the cyclic stress on the elastic fibers causes the elastin fibers to become fractured and separated leading to the stretching and remodeling of the wall associated with thickening of the intima and media, increased collagen content and calcium deposition (Benetos, 1997; Smulyan, 1997). Furthermore, the decreased perfusion to the vasa vasorum observed with aging has been linked to the intrinsic stiffening of aortic tissue (Stefanadis, 1995).

In addition to the associated age related changes in arterial compliance, blood pressure has also been implicated in the modification of arterial structure-function properties (Cameron, 1995). Due to the nonlinear, two-phase (elastin and collagen) viscoelastic nature in the composition of the media of the artery, at low distending pressures, the collagen component is minimally recruited and elastin fibers bear most of the stress. However, at higher pressures, the stiff collagen component is increasingly recruited. This

has important implications for disease processes such as hypertension (O'Rourke, 1992) and atherosclerosis where increases in blood pressure may lead to prolonged and excessive loading on collagen rather than elastin in the maintenance on arterial stucture-function integrity and may result in increased aortic rigidity (Vaitkevicius, 1993).

Physical activity has also been shown to influence arterial wall properties. It has been shown that athletes exhibit greater arterial compliance than sedentary individuals (Kingwell, 1995). In addition, short-term exercise has been demonstrated to increase oxygen consumption as well as arterial compliance (Cameron, 1994). Here, the changes in arterial compliance were attributed to changes in arterial wall properties rather than blood pressure effects, and just as short-term exercise increased compliance, cessation of exercise reduced compliance to normal levels (Cameron, 1994).

In addition to exercise, diet has also been shown to influence arterial stiffness. The major dietary influence on arterial wall properties is salt intake and it has been shown that there is an association between salt intake and arterial stiffness independent of blood pressure (Avolio, 1986). The direct mechanisms by which salt intake influences arterial elasticity are uncertain but it has been proposed that the sympathetic nervous system and the renin-angiotensin-aldosterone system are involved (Draaijer, 1993).

1.6.1.2 Sex hormones and Arterial Compliance

From birth to the onset of puberty, arterial compliance is comparable in males and females. However, from the time of puberty to the onset of menopause, women exhibit greater arterial compliance than age-matched males. Compliance is reduced in

postmenopausal women who are not taking hormonal supplementation and levels are again comparable to those of men. It has been hypothesised that the differences observed between the two sexes is probably attributable to differences in the sex hormones (Laogun, 1982).

Estrogen-containing therapies have been shown to increase arterial compliance in postmenopausal women (Liang, 1997; Rajkumar, 1997). A study by Rajkumar et al. (1997) investigating the effects of estrogen treatment alone versus combined estrogen-progesterone administration found that both treatment groups demonstrated equivalent increases in arterial compliance. The authors of this study concluded that the effects observed were primarily estrogen mediated and proposed two possible mechanisms of action. On the one hand, the observed effects reflected a short-term vasodilatory response in which a transfer of vascular wall stress from collagen to the more distensible elastin fibres occurred leading to more compliant vessels. The second postulation involved long-term changes through structural vascular changes via modulation of vascular smooth muscle cell numbers and relative proportions of collagen and elastin (Rajkumar, 1997).

Androgens have also been suggested to influence the elasticity of large arteries, although to date observations are conflicting. A study by Dockery et al. (2000) demonstrated that androgen deprivation in males is associated with decreased central arterial compliance and reduced central systolic blood pressure (Dockery, 2000). Other studies, however,

have demonstrated potentially negative effects of testosterone on the coronary vasculature (Adams, 1995).

1.7 DHEA - CLINICAL AND EXPERIMENTAL APPROACHES

It has been postulated that declining DHEA levels with ageing are implicated in many age-related diseases, and this has become a focal point of many studies. It has been claimed that DHEA is linked to the pathophysiology of many diseases including coronary heart disease and atherosclerosis, diabetes, immune disorders, malignancies, neurological function, and osteoporosis (for detailed citations see below).

Classically, three approaches have been employed to delineate the actions of DHEA: 1) Correlation of serum levels of DHEA with physiological or pathophysiological conditions, 2) exogenous administration of the hormone to animal or human models and subsequent measurement of physiological or pathophysiological end-points, and 3) in vitro supplementation to cell, tissue, or organ cultures.

1.7.1 Pharmacological and Physiological Actions of DHEA

Most of the current knowledge regarding DHEA action in vivo stems from animal studies, primarily using rodents. However, as rodent adrenal produces very low amounts of DHEA (Hung, 1988) such studies make the interpretation of any observed outcomes difficult when attempting to extrapolate the results and infer parallel conclusions in humans. There are however a significant number of studies using dog, monkey, pig and human models, which add to and aid in the interpretation of these studies. In addition, there are numerous in vitro studies investigating cellular and molecular actions of DHEA. It is therefore the aim of this review to bring into light the current knowledge of

DHEA action in the major areas of study with particular emphasis on the cardiovascular actions of DHEA.

1.7.1.1 Immune Function

Studies of the effects of DHEA on immune function in both animal and human models have yielded conflicting results. Caffrey et al (1994) and Lardy et al (1995) have shown that immunized mice and mice exposed to bacterial polysaccharides exhibit increased antibody production following DHEA treatment (Caffrey, 1994; Lardy, 1995). DHEA administration has also been shown to reverse high-dose antigen-induced immune suppression in mice (Kim, 1995). In human studies, Araneo et al (1995) and Evans et al (1996) reported improvement the immune response in elderly humans following treatment with oral DHEA as an adjuvant in tetanus and influenza vaccination (Araneo, 1995; Evans, 1996). In contrast, a study by Danenberg et al (1997) showed that DHEA treatment did not improve responses to influenza immunization in elderly human subjects (Danenberg, 1997).

DHEA supplernentation reverses age-related cytokine dysregulation and immunosenescence (Spencer, 1995), and in vitro studies have shown the DHEA suppresses pro-inflammatory cytokine secretion from macrophages (Padgett, 1998). A study investigating the ability of DHEA to inhibit the production of inflammatory mediators from glial cells and to change the course of acute central nervous system inflammatory disease in mice and rats found that DHEA selectively inhibits the production of tumor necrosis factor alpha and interleukin-6. However, DHEA

administration did not change the clinical outcome of experimental autoimmune encephalomyelitis (Kipper-Galperin, 1999).

In a study of postmenopausal women, DHEA treatment was found to increase natural killer cell counts and cell cytotoxicity (Casson, 1993). However, the same study also showed a decrease in helper T-cell (CD4+) numbers. In addition, it has recently been shown that DHEA-S increases natural killer cell cytotoxicity in humans via local generation of immunoreactive insulin-like growth factor I (Solerte, 1999).

In a three-month trial, van Vollenhoven et al. (1995) investigated the effects of DHEA administration to a cohort of women with systemic lupus erythmatosis (SLE) and found a significant improvement in the SLE activity index (van Vollenhoven, 1995). Women taking part in this study and receiving DHEA rather than placebo were able to decrease their glucocorticosteroid dose. Interestingly however, Barry and van Vollenhoven (1998) recently showed that clinical response to DHEA in SLE was not clearly dose dependent (Barry, 1998; van Vollenhoven, 1998). Furthermore, serum levels of DHEA and DHEA-S correlated only weakly with lupus outcomes. They concluded that monitoring serum DHEA levels appears to be of limited clinical use.

It has also been reported that oral DHEA administration improves immune function in HIV positive patients (Dyner, 1993), and that low serum DHEA-S levels are associated with HIV illness markers, including viral load (Ferrando, 1999). In addition, a study by Schifitto et al (2000) investigating the relationship between autonomic performance, plasma levels of dehydroepiandrosterone sulfate (DHEA-S), and cytokine profile in HIV

positive patients found that poor autonomic function and low DHEA-S plasma levels tend to be associated with an unbalanced cytokine profile (Schifitto, 2000).

Although many studies have suggested that DHEA improves the immune response in animal and human models, others have shown no such association. It has been shown that DHEA administration to rodents does not retard immune senescence, prevent late life illness (Miller, 1999), or enhance intestinal immune function (Vargas, 1998).

1.7.1.2 Neurological Function

Interpretation of results obtained from studies in rats investigating DHEA action are often complicated because the rodent adrenal produces very low amounts of DHEA (Hung, 1988) and circulating levels are almost immeasurable. In contrast, DHEA is readily synthesized in the brain of rats and has been shown to act on neuroreceptors (Young, 1991).

Evidence for DHEA-specific effects on neural and cognitive function primarily stems from animal studies showing that DHEA influences hypothalamic neurotransmission and eating behavior (Porter, 1995; Porter, 1995). The effects of DHEA on the central nervous system are thought to be mediated through DHEA/GABA-receptor interactions whereby DHEA acts as a potent receptor antagonist (Majewska, 1990). There is also some evidence that DHEA sulphate functions as a sigma-1 receptor agonist (Monnet, 1995), resulting in increased neuronal excitability.

A study by Young and colleagues showed that long-term administration of DHEA reduced aggression in androgenised female mice (Young, 1991). It has been suggested that DHEAS may potentially enhance memory by facilitating the induction of neural plasticity (Diamond, 1996), or by its actions on GABA receptors since it has been shown that learning and memory are enhanced by GABA receptor antagonism. While these and other studies have shown that DHEA reduces anxiety and aggression, and increases memory in rodents (Flood, 1988; Flood, 1988; Melchior, 1994), the evidence is less clear-cut in human models.

Several studies have reported an improvement in mood and "well being" in humans following DHEA administration (Morales, 1994; Wolkowitz, 1995). It has also been shown that DHEA administration at night enhances rapid eye movement (REM) sleep patterns (Friess, 1995). A recent study demonstrated that DHEA replacement corrects the steroid deficiency seen in Addison's disease and improves some aspects of psychological function (Hunt, 2000). Other studies have, however, report no beneficial effects on mood, well being or cognition in response to DHEA supplementation (Vogiatzi, 1996; Wolf, 1997). Further, it has been shown that DHEA administration to postmenopausal women may result in increased levels of some of the components seen in neurofibrillary tangles of Alzheimer's Disease (Hashimoto, 1997).

1.7.1.3 Body Composition

Another arena of research concerning DHEA action is that of body composition and obesity. The general hypotheses concerning potential DHEA effects on body composition stem from a study conducted by Yen et al. showing that DHEA

supplementation to genetically altered mice resulted in significant weight reduction without altering food consumption compared to mice fed the same diets without DHEA (Yen, 1977). Although the authors postulated that the mechanism behind the observed weight reduction was an increase in metabolism, studies that followed suggested that DHEA may be thermogenic (Tagliaferro, 1986). Weight reducing effects of DHEA have also been reported in spontaneously obese dogs (Kurzman, 1990).

Another possible mechanism by which DHEA may affect weight is through the reduction of appetite. A study by Abadie et al. (1993) showed that there was a significant weight loss with decreased food intake in obese Zucker rats fed a DHEA supplemented diet. They also demonstrated that the DHEA fed rats exhibited higher levels of serotonin in the hypothalamus compared to rats fed a regular diet, suggesting a central effect of DHEA on appetite suppression (Abadie, 1993). Subsequent studies in rodents have also reported similar reductions in appetite when fed a DHEA supplemented diet (Porter, 1995; Richards, 1999). Other studies have, however, shown little or no effect of DHEA on altering appetite. In a study conducted on rhesus monkeys, it was found that DHEA transiently altered food intake, characterized by an initial increase followed by a decrease in appetite (Christopher-Hennings, 1995).

Although most of the current evidence supports beneficial effects of DHEA on body composition and inhibitory effects on food intake in animals, studies conducted on humans have yielded conflicting outcomes. An early study conducted by Nestler et al. showed a substantial reduction in body fat, an increase in muscle mass, and a reduction in serum low density lipoprotein cholesterol levels in lean men following twenty-eight

days of DHEA supplementation (Nestler, 1988). Other studies have also reported DHEA associated alterations in body fat mass in elderly men and postmenopausal women (Walker, 1999; Villareal, 2000), Abbasi et al. (1998) reported a significant correlation between serum DHEA levels, body fat content and percent in lean body mass in men but not in women (Abbasi, 1998).

Most studies, however, have failed to demonstrate any significant effect on weight (Usiskin, 1990) or body composition (Welle, 1990; Morales, 1994). A recent double blind, placebo-controlled study investigating the effects of long term DHEA treatment on body composition and serum leptin levels in women with adrenal insufficiency showed no significant effects of DHEA on carbohydrate metabolism or body composition. There were, however, changes in serum leptin levels (Callies, 2001).

1.7.1.4 Bone Metabolism

As mentioned previously, ageing is accompanied by marked changes in bone mineral density. It has therefore been suggested that declining DHEA levels seen in ageing play a role in the progression of osteoporosis. Indeed, studies have suggested that DHEA supplementation prevents bone loss and in some cases increases bone mineral density. A recent study in ovariectomized rats showed that DHEA supplementation reversed the ovariectomy-induced decreases in bone formation and strength (Higdon, 2001). Two interventional studies investigating the effects of DHEA on bone formation in postmenopausal women showed that DHEA increases bone mineral density and parameters of bone formation and turn over (Labrie, 1997; Labrie, 2001). While studies have suggested a beneficial role for DHEA in the setting of osteoporosis in

postmenopausal women, studies in men have found no improvement in parameters of bone formation following DHEA administration, suggesting gender-dependent differences in the manner in which DHEA might affect bone formation (Callies, 2001).

1.7.1.5 Malignancy

One of the most controversial areas of DHEA research is its potential role in malignancy biology, for, just as many studies have shown inhibitory effects of DHEA on the progression of cancers, an equal number have demonstrated deleterious DHEA effects. The evidence also varies according to the type and location of the observed malignancy.

Studies conducted in the early 1990s by Rao et al. demonstrated that while DHEA inhibits testicular malignancies in rats, it also stimulates hepatic cancer growth (Rao, 1992; Rao, 1992). Subsequent studies have also raised concerns regarding the stimulation of hepatic cancers by DHEA (Sakuma, 1992; Prough, 1995). It is important to note that these observations were made in rodent models and that how they relate to hepatic tumors in humans is not yet clear. Further, the doses used in studies showing potentially deleterious DHEA effects on the liver are markedly higher than those demonstrating inhibitory effects of DHEA on cancers in several rodent tumorigenesis models (Schwartz, 1995).

The proposed mechanisms for the anti-tumor effects of DHEA in animal models include DHEA-induced inhibition of glucose 6-phosphate dehydrogenase (G-6-PD), or modulation of cytokines (Schwartz, 1986). DHEA administration has been shown to inhibit G-6-PD and the pentose phosphate pathway in mouse embryo and human

lymphocyte and skin fibroblast cultures (Gordon, 1987; Shantz, 1989; Feo, 1991), and aid in cell resistance to transformation by carcinogens (Nyce, 1984). Inhibition of G-6-PD results in a suppression of NADPH and ribose-5-phosphate, which is necessary for DNA synthesis (Schwartz, 1986; Gordon, 1987; Spencer, 1995). A further important insight into the mechanisms of DHEA action concerning hepatic carcinoma was the observation that DHEA only acts in animals that have functional PPAR-alpha receptors, and may therefore serve as an important endogenous regulator of liver peroxisomal enzyme expression (Peters, 1996).

Epidemiological studies in humans have also supported the notion that high doses of DHEA may have stimulatory effects on cancer progression. Helzlsouer et al. reported an increased incidence of ovarian cancer in women with higher base-line levels of DHEA compared to cancer-free controls in a fifteen year follow-up study (Helzlsouer, 1995). Further, it has been shown that higher levels of circulating DHEA are associated with an increased incidence of breast cancer development in postmenopausal women (Gordon, 1990). Conflicting observations have been reported by Barrett-Connor who showed no association between DHEA levels and breast cancer development in postmenopausal women (Barrett-Connor, 1990). Furthermore, in premenopausal women, circulating DHEA/DHEAS levels have been found not to be associated with breast cancer development (Helzlsouer, 1992).

Other studies have shown little or no association between plasma DHEA levels with other carcinoma types. Alberg et al. reported that there is little association between circulating DHEA and colon cancer risk although in men, DHEA may in fact reduce the

risk of colon cancer (Alberg, 2000). These findings are coupled with those of another study showing no association between serum DHEAS levels and thyroid cancer risk (Berg, 1999). Other studies, however, have suggested that DHEA may increase the risk of developing prostate cancer in rodent models (Labrie, 1989; Schiller, 1991).

In vitro studies have also presented conflicting observations with respect to the possible involvement of DHEA in inducing or retarding carcinoma progression. A recent study demonstrated that DHEA stimulates the proliferation of MCF7SH-breast cancer cells in culture (Maggiolini, 1999). Another study, however, showed that DHEA inhibits human breast epithelial carcinoma cell proliferation via a post-transcriptional mechanism (Ciolino, 1999).

1.7.1.6 Cardiovascular Health

The potentially beneficial effects of DHEA on cardiovascular health were first suggested in an early study showing that levels of DHEA and its urinary metabolites, 17 ketosteroids, were lower in men with atherosclerotic disease as determined postmortem (Kask, 1959). Observations from animal studies conducted in the late 1980s and early 1990s have further added to this viewpoint. In a parallel study, DHEA administration to hypercholesterolaemic rabbits with provoked endothelial injury resulted in a 50% reduction in atherosclerotic plaque size compared to rabbits fed a DHEA-depleted diet (Gordon, 1988). Another study investigating the effects of DHEA in hypercholesterolaemic rabbits found that DHEA administration reduced aortic fatty streak formation by up to 40% (Arad, 1989). Coupled with these studies and demonstrated once again in a rabbit model, a significant retardation in the progression of

atherosclerosis in response to chronic DHEA administration has been reported (Eich, 1993).

Human epidemiological studies, however, have not supported the results of these animal studies. The conflicting observations reported between the various human studies primarily stem from gender differences as well as from the nature of the end-points considered, the most obvious of which is mortality. A positive relationship between DHEA/DHEAS levels and cardiovascular health was established in a study comparing DHEA levels in men who died of coronary artery disease to those who survived (LaCroix, 1992). Other studies investigating fatal cardiovascular events and ischaemic heart disease also divulged an inverse relationship between DHEAS levels and fatal coronary disease (Barrett-Connor, 1986; Barrett-Connor, 1995).

Conversely, studies investigating non-fatal coronary end points have shown little or no association between DHEA/DHEAS levels and myocardial infarction or coronary artery disease (LaCroix, 1992; Newcomer, 1994). Furthermore, there has been no epidemiological association between DHEA/DHEAS levels and coronary end-points, whether fatal or not, in female cohorts (Barrett-Connor, 1995).

The inherent flaw common to all the epidemiological studies mentioned is the reliability of single hormonal measures used to characterize individuals. In the aforementioned studies, DHEAS levels are quoted rather than DHEA, as DHEA levels tend to show more variation than DHEAS due to differences in diurnal variations as well as clearance

rates and half-life lengths (Rosenfeld, 1971; Nieschlag, 1973; Baulieu, 1996; Khorram, 1996).

Other evidence supporting possible beneficial DHEA-effects on the cardiovascular system comes from cross-sectional studies, which show significantly reduced levels of DHEA/DHEAS in men with previous coronary events compared to those without a history of coronary disease (Slowinska-Srzednicka, 1989; Herrington, 1990; Mitchell, 1994; Feldman, 1998). Comparable studies, however, have failed to confirm these findings. A study paralleling that of Herrington et al., which suggested a positive dose-response relation between plasma DHEAS levels and angiographically defined coronary atherosclerosis in men, showed that there was no significant relationship between DHEAS levels and coronary artery disease (Hauner, 1991). Furthermore, other studies have even suggested that increased DHEAS levels may potentially have negative effects on coronary end-points in men with prior myocardial infarction (Zumoff, 1982; Hautanen, 1994).

Few studies have investigated the effects of exogenous DHEA administration to humans with regards to cardiovascular health. Mattson et al. investigated the effects of intramuscular DHEA supplementation in young ovariectomised women and reported a reduction in total and HDL cholesterol levels (Mattson, 1980). These observations were confirmed by Mortola et al. who reported similar reductions in postmenopausal women (Mortola, 1990). However, another study demonstrated reductions in total and LDL cholesterol with no changes in HDL cholesterol in response to oral administration to young healthy men (Nestler, 1988). It is important to note, however, that the doses used

in the two latter studies were eight-fold greater than those previously used (1600 mg Vs 200 mg daily).

Although relatively few studies have investigated the relationship between DHEA/DHEAS levels and cardiovascular health in women, current evidence points out an apparent contrast between the possible protective role of this hormone in men but no or even negative effects in women. Because DHEA is a precursor to both androgens and estrogens it has been proposed that DHEA can have a duel action in the different genders according to the hormonal environment. In men, for example, DHEA may act as an estrogen and accordingly provide cardioprotection, whereas in women, DHEA may act as an androgen and subsequently increase the risk of cardiovascular disease (Ebeling, 1994). It is difficult to substantiate these theories as no study has investigated the direct effects of exogenous DHEA administration on physiological cardiovascular end-points in either gender.

1.7.1.6.1 Possible mechanisms of DHEA action on cardiovascular physiology

The precise modes of action of DHEA in cardiovascular physiology remain unresolved, although studies are increasingly investigating possible in vitro and in vivo mechanisms. It is well recognized that sex hormones, and in particular estrogens, can affect the lipid profile, and as DHEA is a natural precursor to both androgens and estrogens, it has been hypothesized that DHEA may exert its actions on the cardiovascular system through its effects on lipid metabolism. Although several studies have reported alterations in lipid parameters following DHEA administration, the results remain contradictory, ranging from beneficial effects (Nestler, 1988; Kurzman, 1990; Haffner,

1995) to negative effects (Mattson, 1980; Mortola, 1990; Bednarek-Tupikowska, 1995). Several studies have also reported no effects of DHEA on lipid metabolism (Gordon, 1988; Arad, 1989; Eich, 1993). A recent in vitro study demonstrated that DHEA reduces lipid peroxidation of platelet membranes in the presence of iron, while cholesterol increases peroxidation (van Rensburg, 2000).

In vitro studies have shown DHEA-induced inhibition of aortic vascular smooth muscle cells as well as inhibition of MAP-kinase phosphorylation (Yoneyama, 1997; Yoshimata, 1999), which could be implicated in the retardation of the pathogenesis of atherosclerosis (Herrington, 1995). These results complement an earlier report by Saenger et al. who demonstrated the inhibitory effects of DHEA on fibroblast cell proliferation (Saenger, 1977). Platelet aggregation and plasminogen activator inhibitor type 1 are also reduced by DHEA administration (Jesse, 1995; Beer, 1996).

In vivo studies have demonstrated beneficial effects of DHEA on progressive dermal ischaemia caused by thermal injury (Araneo, 1995) as well as muscle microcirculation following ischaemic damage (Lohman, 1997), suggesting that DHEA may affect vascular reactivity. This hypothesis is further supported by studies demonstrating that DHEA increases NO synthesis in human pregnancy (Manabe, 1999) and retards atherosclerosis via NO mediated mechanisms (Hayashi, 2000).

Although a number of studies have suggested a potentially beneficial role for DHEA in cardiovascular physiology the precise mechanism(s) of DHEA action remain elusive. Several plausible hypotheses have been put forward address this issue: (i) The partial

conversion of DHEA into androgens and estrogens and subsequent action of these hormones via known classical pathways, (ii) DHEA exerts physiological effects in its own right via direct androgen/estrogen receptor mediated mechanisms, (iii) DHEA has its own unique biological actions mediated through non-classic steroid receptors. Identifying the precise mechanism(s) of DHEA action in cardiovascular tissue would undoubtedly lend credence to this investigative area.

It is therefore the aim of this project to investigate the effects of DHEA on the cardiovascular system using both in vitro and in vivo approaches. We sought to examine the effects of DHEA on cultured VSMC and endothelial cell proliferation, and to elucidate the possible cellular and regulatory pathways through which DHEA might be exerting its effects. Clinically, we investigated the effects of exogenous DHEA supplementation on parameters of cardiovascular risk, and in particular mechanical arterial properties and blood pressure, and markers of endothelial function, in healthy postmenopausal women.

DHEA & The Vasculature

~Experimental Approaches~

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Chapter 2

DHEA Action in Human Vascular Smooth Muscle Cells: Proliferation, Receptors and Subcellular Mechanisms

2.1 INTRODUCTION

DHEA is quantitatively the most abundant circulating adrenal steroid and has been the subject of many studies. However, the possibility of its having biological actions in its own right has been largely overlooked, in that it has generally been assumed that its actions reflect conversion to androgenic and estrogenic metabolites. Evidence in favor of this assumption includes the presence of the enzymes responsible for 3β oxidation of DHEA to androstenedione (3β-HSD), for 17β reduction of androstenedione to testosterone and estrone to estriol (17β-HSD), and for aromatization of androgens into estrogens (P450 aromatase) in many peripheral tissues, and perhaps also in vascular smooth muscle cells (VSMC) (Ebeling, 1994; Labrie, 1995).

In vivo evidence in support of DHEA conversion to androgens and estrogens includes a study investigating the metabolism of DHEA in a cohort of patients with panhypopituitarism (Young, 1997) which showed that DHEA administration induced a significant, dose dependent increase in plasma estrogens, particularly estradiol. The authors of this study concluded that the biotransformation of DHEA into potent androgens and estrogens may explain several of the reported beneficial actions of this steroid in aging people. Another study investigating the effects of DHEA replacement in women with adrenal insufficiency found that DHEA administration increased serum levels of DHEA, DHEAS, androstenedione and testosterone (Arlt, 1999). These studies have been complemented by others showing that DHEA administration results in increased levels of androgens and estrogens (Haning, 1991; Haning, 1991).

VSMC proliferation is an important component of the remodeling of blood vessels and has been implicated in the pathogenesis of atherosclerosis (Chapter 1.5.1). VSMC proliferation and migration are modulated by a variety of paracrine and haemodynamic factors (Chapter 1.5.1) and accelerated in disease states such as diabetes (Faries, 2001), hypertension and atherosclerosis (Li, 2000).

Early studies in animals have shown a reduction in the occurrence and progression of atherosclerosis following estrogen administration (Adams, 1990; Kushwaha, 1991). In addition, epidemiological studies have suggested that estrogen-containing therapies reduce the incidence of CHD in postmenopausal women (Stampfer, 1991; Nabulsi, 1993). Such studies have led to investigations into the possible mechanisms by which estrogens, and sex hormones in general, might beneficially contribute to cardiovascular health. While studies have demonstrated that estrogens improve the lipid profile by reducing LDL cholesterol (Wagner , 1991) and increasing HDL cholesterol (Stevenson, 1993; Stevenson, 1998), there is increasing evidence that sex hormones have direct effects on the behavior of cells that comprise the vessel wall, namely endothelial and smooth muscle cells (Chapter 1.3.1.4 and 1.4.2).

Estrogens have been shown to inhibit arterial VSMC proliferation (Vargas, 1993; Bhalla, 1997) and migration (Kolodgie, 1996) by receptor mediated mechanisms (Somjen , 1998), and therefore possibly to prevent atherosclerosis (Morey, 1997). The subcellular mechanisms by which estrogens beneficially influence VSMC proliferation include the down-regulation of the transcription factor SP-1 (Ling, 2001), activation of cyclic AMP-adenosine pathways (Dubey, 2000), and inhibition of MAP-kinase activity (Morey, 1997).

While the effects of estrogens on VSMC behavior are currently under intense investigation, the role of androgens in vascular biology has attracted relatively little attention. Furthermore, studies investigating the effects of androgens have yielded conflicting results. It has been shown that testosterone increases the proliferation of VSMC by receptor-mediated mechanisms (Gerdes, 1996; Fujimoto, 1994), and therefore may contribute to the progression of atherosclerosis. However, a recent study by Somjen et al. has shown inhibitory effects of testosterone and dihydrotestosterone on umbilical VSMC proliferation (Somjen, 1998), a potentially beneficial effect in the setting of cardiovascular physiology.

The apparent opposing effects of androgens and estrogens on VSMC proliferation have further stimulated interest in the potentially beneficial effects of DHEA on cardiovascular pathophysiology. Studies have suggested that DHEA attenuates VSMC proliferation (Yoneyama, 1997; Dashtaki, 1998; Furutama, 1998). However, as prolonged pretreatment periods with DHEA are required before an effect is observed, it has been suggested that the actions of DHEA may result from the production and subsequent action of estrogenic or androgenic metabolites arising from its conversion (Yoneyama, 1997). In addition, the possibility of specific receptors and subcellular mechanisms whereby DHEA might potentially exert vascular effects has not been explored.

Aims of Study

To address the question of the potentially beneficial effects of DHEA on VSMC behavior this study sought to examine the actions of DHEA on the proliferation of

cultured VSMC and on the potential signaling pathways through which its effects might be mediated.

Further, to investigate whether the effects of DHEA on VSMC proliferation are mediated via DHEA conversion to androgens or estrogens, or by direct DHEA action, the effects of DHEA on VSMC proliferation in the presence of androgen and estrogen receptor antagonists, was examined. DHEA binding studies were also performed to investigate the possible existence of specific DHEA receptor in these cells.

Part A

DHEA Inhibits Human Vascular Smooth Muscle Cell Proliferation Independently of Androgen and Estrogen Receptors

2A.1 METHODS

2A.1.1 Experimental protocol

Internal mammary artery (IMA) VSMC were kindly donated by Dr. Peter J. Little of the Baker Medical Research Institute, Melbourne Australia. Cells were harvested from the target vessels of four donors (sex unknown) by an explant technique. A segment of the IMA was cut out and placed into ice-cold Dulbecco's modified Eagle's medium (DMEM). All external fat and connective tissue were microscopically cleaned from the vessel.

Following removal of the endothelial layer, the IMA was cut longitudinally, and the remaining strips were transferred to 60-mm dishes with DMEM in the presence of 5 mmol/L glucose and 10% fetal bovine serum (FBS), and placed into an incubator of 5% CO_2 at 37 °C. Culture media were changed every three days. Following migration from the vessel strips, the cells were allowed to proliferate and subsequently subcultured to near confluence. Smooth muscle cells were characterized by incubation with smooth

muscle α-actin followed by immunofluorescence staining and Western blot analysis. Cells at passages 11-15 were used in the study.

2A.1.2 Cell proliferation

Cell proliferation was determined by a [^{3}H]-thymidine incorporation assay measuring DNA synthesis. Cells were seeded in 24-well plates (Falcon, Becton Dickinson Labware)(10, 000 cells/ml) and grown to 80-90% confluence in DMEM with 10% FBS. Following serum deprivation for 24 h, the cells were treated with either DHEA, 17β-estradiol, androstenedione or testosterone (Sigma) (0.1 – 100 nM) for 4h, and subsequently with the hormones and platelet derived growth factor BB (PDGF BB) (Sigma) (10ng/ml) together for 20 h.

2A.1.2.1 [^{3}H]-Thymidine incorporation

[^{3}H]-Thymidine 1 μCi/well (ICN Biomedicals) was added during the last 3 h of PDGF BB treatment, the cells washed twice with ice-cold Dulbecco's PBS, and incubated then with ice-cold 0.2 N $HClO_4$ (1 mL/well) on ice for 30 min. After washing (0.5 mL/well, ×3) with 0.2 N $HClO_4$, cells were incubated with 0.5 mL/well of 0.2N NaOH in 37 °C for over 1 h, which was then neutralized with 0.2 mL/well of 6% acetic acid. The contents of each well were transferred into scintillation vials with Instagel (3 mL) (Instagel, BIO-RAD, Australia) and counted for 2 min per vial in a β-counter.

2A.1.3 Pharmacological antagonism of estrogen (ER) and androgen (AR) receptors

To determine whether the effects of DHEA on cell proliferation are mediated via ER or AR, measurements of cell proliferation were performed as described above in the presence and absence of the ER antagonist ICI 182,780 (Tocris Cookson Inc. USA) or the AR antagonist flutamide (Sigma, USA) added 2 hrs prior to the addition of the various hormones.

2A.1.4 Statistical analysis

All data are presented as mean ± the standard error of the means (SEM). Statistical analysis between two observations was by Student's t-test and for multiple comparisons by analysis of variance. The null hypothesis was rejected at $p>0.05$.

2A.2 RESULTS

2A.2.1 Effects of DHEA on VSMC proliferation

Both DHEA and 17β-estradiol (0.1-100 nmol/L) attenuated PDGF BB-induced increases in DNA synthesis in VSMC in a dose dependent manner, with a maximum inhibitory effect of DHEA to 61±3% and 17β-estradiol to 53±4% compared with control. Conversely, testosterone at the same concentrations enhanced DNA synthesis, as did androstenedione (167±9% and 131±3% of control respectively)(Fig. 2A.1). These results indicate that DHEA inhibits PDGF BB-induced VSMC proliferation in a fashion similar to estrogen and in a direction opposite to that of testosterone and androstenedione.

2A.2.2 Effects of the AR antagonist flutamide and the ER antagonist ICI 182,780 on DHEA-induced inhibition of VSMC proliferation

The inhibitory effect of DHEA (10 nmol/L) on PDGF-BB induced cell proliferation was not affected by either the AR antagonist flutamide or the ER antagonist ICI 182,780 (100 nmol/L). Flutamide completely abolished the stimulatory effects of testosterone, and ICI 182,780 blocked the inhibitory effects of 17β-estradiol on the cells (Fig 2A.2), evidence that the actions of DHEA on VSMC proliferation are not mediated by either AR or ER. Flutamide and ICI 182,780 alone had no proliferative effects on the cells.

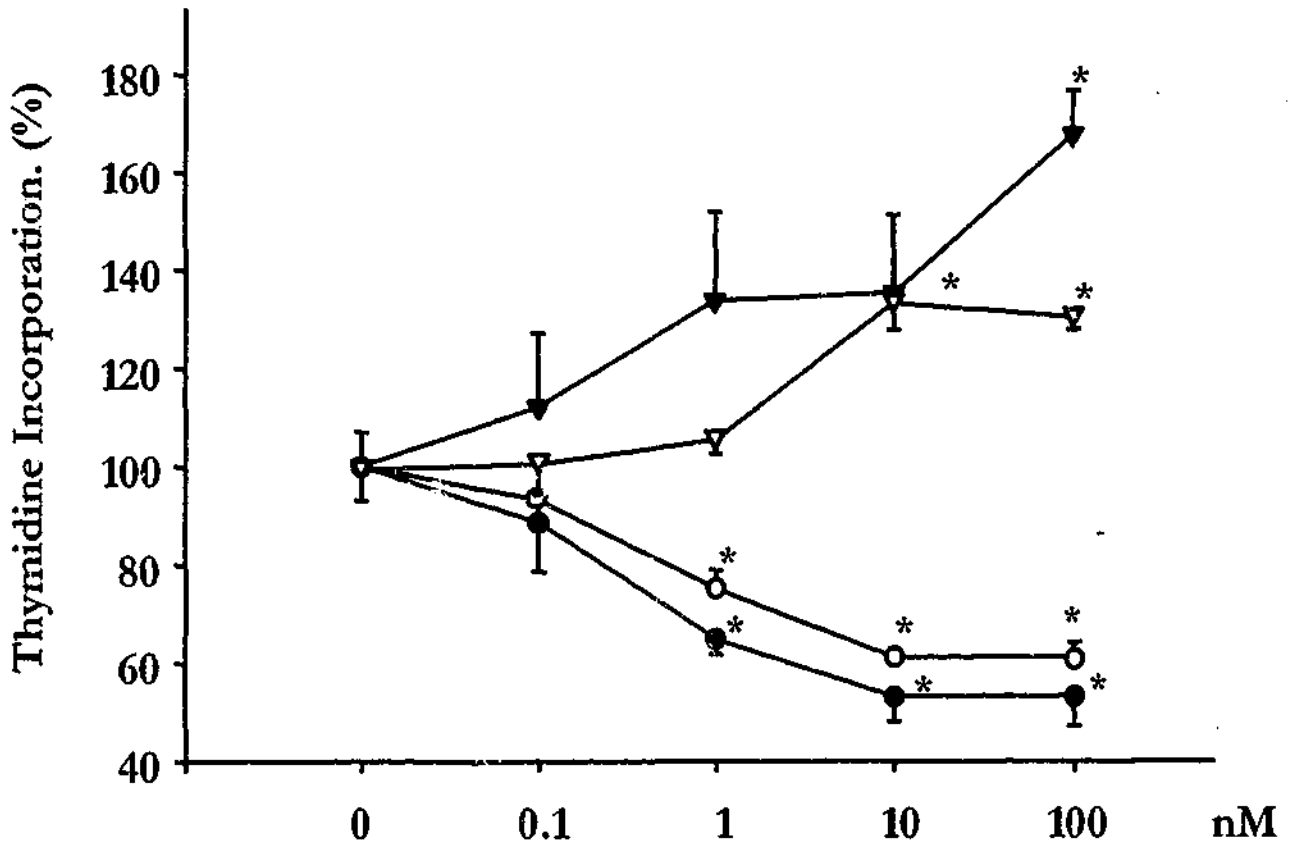


Figure 2A.1 Effects of DHEA, 17β-estradiol, testosterone and androstenedione on PDGF-BB induced VSMC proliferation

DHEA (—○—) and 17β-estradiol (—●—) (0.1-100 nmol/L) attenuate PDGF BB-induced increases in DNA synthesis in VSMC in a dose dependent manner, with a maximum inhibitory effect of DHEA to 61±3% compared with 17β-estradiol (53±4% of control). Testosterone (—▼—) and androstenedione (—▽—) increase DNA synthesis (167±9% and 131±3% of control respectively).

Data are presented as mean percentage of PDGF-BB stimulation of DNA synthesis ± SEM.
* denotes a significant difference from PDGF-BB induced increases in DNA synthsis in VSMC.
n = 6, with 4 replicates in each experiment

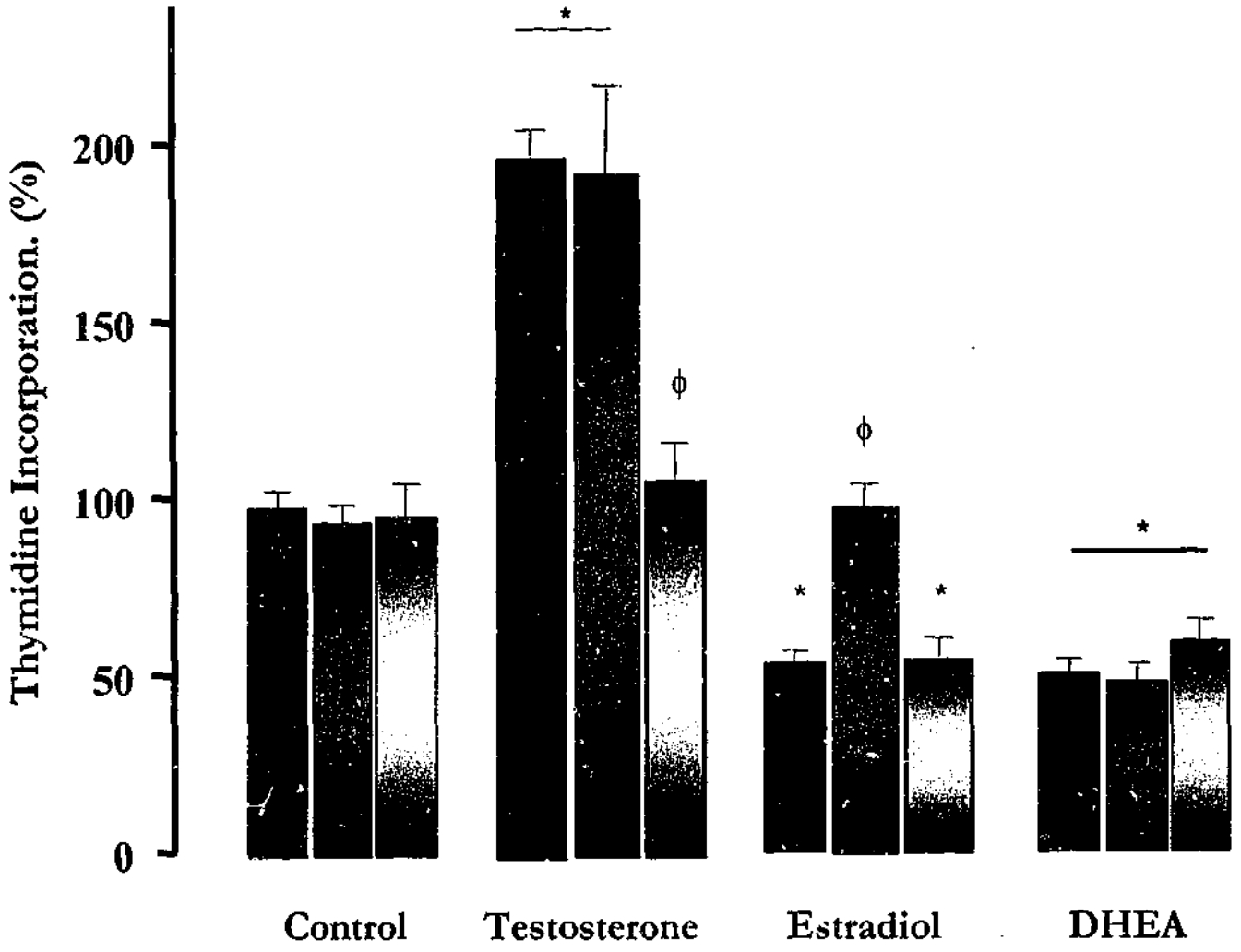


Figure 2A.2 The effects of the AR antagonist flutamide and the ER antagonist ICI 182,780 on DHEA-induced inhibition of VSMC proliferation

The AR antagonist flutamide (100 nmol/L) completely abolishes the stimulatory effects of testosterone (10 nmol/L), and the ER antagonist ICI 182,780 blocks the inhibitory effects of 17β-estradiol, but neither antagonist affects the inhibitory actions of DHEA on VSMC proliferation. Human VSMC were pretreated with androgen or estrogen receptor antagonists (100 nmol/L, 2h) prior to exposure to steroids (10 nmol/L)

Data are presented as mean percentage of PDGF-BB stimulation of DNA synthesis ± SEM

* denotes a significant difference from PDGF-BB induced increases in DNA synthesis

ϕ denotes a significant difference from the hormone-alone treatment.

n = 5, with 4 replicates in each experiment

Hormone alone

Hormone + ICI 182 780

Hormone + Flutamide

2A.3 DISCUSSION

To our knowledge, this is the first study to provide direct evidence that the hormone DHEA attenuates human VSMC proliferation by mechanisms independent of either AR or ER. DHEA and 17β-estradiol attenuated PDGF-BB induced proliferation, whereas testosterone and androstenedione significantly stimulated cell proliferation. While the inhibitory effects of estradiol and the stimulatory effects of testosterone were completely abolished by ER and AR antagonists respectively, neither receptor antagonist influenced the inhibitory effects of DHEA on VSMC proliferation.

The possibility of DHEA exerting vascular effects directly has been increasingly canvassed but the studies to date have yielded conflicting results. Bruder et al reported that DHEA stimulates the estrogen response element independently of conversion to estrogens (Bruder, 1997). However, as this effect was blocked by the specific ER antagonist ICI 182,780 it suggests that DHEA may have been acting via estrogen receptors or that the ICI compound also blocked the putative DHEA receptor; in addition, the possibility of DHEA conversion to other, androgenic metabolites cannot be excluded in this study.

Although estrogen-like effects of DHEA have been observed both *in vitro* and *in vivo*, androgenic effects have also been demonstrated. Sourla et al showed that DHEA had androgenic effects on rat mammary gland histomorphology and structure, which were abolished by the specific AR antagonist flutamide but not by the ER antagonist EM-800. The findings of this study thus suggest that in the rat mammary gland DHEA exerts its

effects via the AR (Sourla, 1998). They do not, however, exclude the possibility of DHEA conversion to androgens, and therefore do not constitute convincing evidence for a specific DHEA action independent of conversion.

The findings of the current study showing that DHEA inhibits the proliferation of human IMA VSMC are consistent with those previously reported in other cell lines, such as human aortic VSMC (Yoneyama, 1997), fibroblasts (Saenger, 1977), T-lymphocytes (Meikle, 1992), and preadipocytes (McIntosh, 1998). However, the present study has also demonstrated that the inhibitory effects of DHEA on VSMC proliferation are independent of both AR and ER, evidenced by the DHEA-induced response being unaffected by either flutamide or ICI 182,780. In contrast, the inhibitory effect of 17β-estradiol and the stimulatory effect of testosterone were completely abolished by their respective receptor antagonists.

These results strongly suggest that while DHEA has an estrogen-like effect on VSMC proliferation, its mode of action is unlike that of either 17β-estradiol or testosterone. The demonstration that androstenedione, the principal product of DHEA conversion in human VSMC, showed stimulatory effects on cell proliferation (that is, opposite to those seen with DHEA), is further evidence that the observed effects are due to a direct interaction rather than conversion.

Part B

DHEA-Specific Binding Sites in Human Vascular Smooth Muscle Cells

2B.1 METHODS

2B.1.1 Estrogen receptor (ER) and androgen receptor (AR) studies

ER and AR density was determined by radioligand binding assay. Cells were seeded and allowed to grow to confluence on 6-well plates (~10×10^5 cells per well) and incubated with [^{3}H]-estradiol (0.315-5.0 nmol/L) with or without nonradioactive diethylstilbestrol (DES, 1 μmol/L) for ER, and [^{3}H]-R1881 (0.31-5 nmol/L) with 1 μmol/L triamcinolone acetomide (TA) plus or minus nonradioactive DHT (1 μmol/L) for AR, for 90 min. Cells were washed with D-PBS and scraped out in the presence of 0.1% trichlor acetic acid (TCA) (1.5 mL/well). Extracts were put into scintillation vials with 5 mL scintillation liquid for counting (5 minutes per vial) in a β-counter. Radioligand binding assays were kindly carried out by Dr Shanhong Ling and Kathy Myles.

ER and AR protein expression was analyzed by Western blot. Cells were washed with ice-cold PBS (×2) and lysed by incubation on ice for 30 minutes with lysis buffer (20 mmol/L Tris-base pH7.7, 250 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA,

0.5% NP-40, 10% glycerol, 20 mmol/L β-glycerophosphate, 1 mmol/L Na-vanadate). Leupeptin (10 µl/ml), 5 µl/ml aprotinin, 1 µmol/L pepstatin, 1 mmol/L AEBSF and 10 mmol/L DTT were added before use. Total proteins were isolated by centrifuging at 5,000 rpm for 15 min, and 30 µg protein electrophoresed on 10% SDS-polyacrylamide gels and transferred to Hybond ECL filters (Sigma, MI, USA). The filters were blocked with 5% nonfat dry milk in TBS (20 mmol/L Tris, pH7.5, 50 mmol/L NaCl, and 0.1% Tween-20) overnight, then washed and incubated with primary antibodies against ERα, ERβ or AR (Santa Cruz Biotechnology, Inc., CA, USA) for 1 h. After washing (10 min x 3), blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (DAKO, DK, Denmark) for 1 h, washed (10 min x 3), incubated for 1 min with enhanced chemiluminescence reagents (Amersham Corp.), and exposed to X-ray film. For protein loading controls, the blots were washed again and probed with an anti-human smooth α-actin antibody (DAKO Corp) by the method described above. For relative quantification, bands were scanned on a PowerLook Scanner (Industrial Park, Taiwan).

2B.1.2 ER and AR binding assays

Competitive binding assays were carried out to determine the relative binding affinity of DHEA for ER and AR. Confluent monolayers of cells in 6-well plates were exposed to DHEA (1-1000 nmol/L) plus 1 nmol/L of either [^{3}H]-estradiol or [^{3}H]-R1881, with appropriate concentrations of testosterone or 17β-estradiol as controls. Following incubation at 37 ^{0}C for 60 min, cells were washed with D-PBS, treated with 0.1% TCA

and harvested. Extracts were transferred to scintillation vials with 5 mL scintillation liquid for counting (5 min per sample) in a β-counter.

2B.1.3 DHEA binding assays

Similar competitive binding assays were carried out to explore DHEA binding to intact cells. Confluent monolayers of cells in 6-well plates were treated with [^{3}H]-DHEA (10 nmol/L) and 1-10,000 nmol/L non-radioactive DHEA. Following incubation at 37 ^{0}C for 90 min, cells were washed with D-PBS and bound DHEA was determined as above.

Scatchard analysis was carried out to determine the affinity and capacity of DHEA binding. Confluent monolayers of cells in 6-well plates were treated with [^{3}H]-DHEA (49-1.5 nmol/L) in the presence or absence of 10 μmol/L unlabeled DHEA. Following incubation at 37 ^{0}C for 90 min, cells were washed with D-PBS and bound DHEA was determined as above. Kd was determined as the gradient of the scatchard, and the number of binding sites by dividing the x intercept by the total cell number. Cell number was determined as previously detailed.

2B.1.4 Statistical analysis

All data are presented as mean ± the standard error of the means (SEM). Statistical analysis between two observations was by Student's t-test and in multiple comparisons by analysis of variance. The null hypothesis was rejected at $p>0.05$.

2B.2 RESULTS

2B.2.1 Presence of AR and ER

Western blot analysis confirmed the expression of AR and ER protein (Fig 2B.1). Radioligand binding assays confirmed the presence of both AR and ER in these VSMC at the density of ~20,000 sites/cell (Kd = 0.44 nmol/L) for AR (Fig 2B.2) and ~24,000 sites/cell (Kd = 0.51 nmol/L) for ER (Fig 2B.3).

2B.2.2 DHEA Binding to AR and ER

DHEA at concentrations of 1 μmol/L showed approximately 5% the potency of testosterone in binding to AR as shown by the displacement of 1 nM [^{3}H]R1881 (Fig 2B.4), and no specific binding to ER (Fig 2B.5.).

2B.2.3 Specific DHEA Binding

The presence of saturable binding sites for DHEA was demonstrated by the ability of nonradioactive DHEA to compete for binding with [^{3}H]DHEA (10nM); non-specific binding (in the presence of 10 μM DHEA) varied from 50% to 65% of total (Fig 2B.6a). Scatchard analysis of specific [^{3}H]DHEA (1.4-49 nM) binding with and without 500-fold excess of nonradioactive DHEA showed that there were ~37,000 binding sites/cell, binding [^{3}H]DHEA with an affinity given by Kd 37°C 14 nM (Fig 2B.6b).

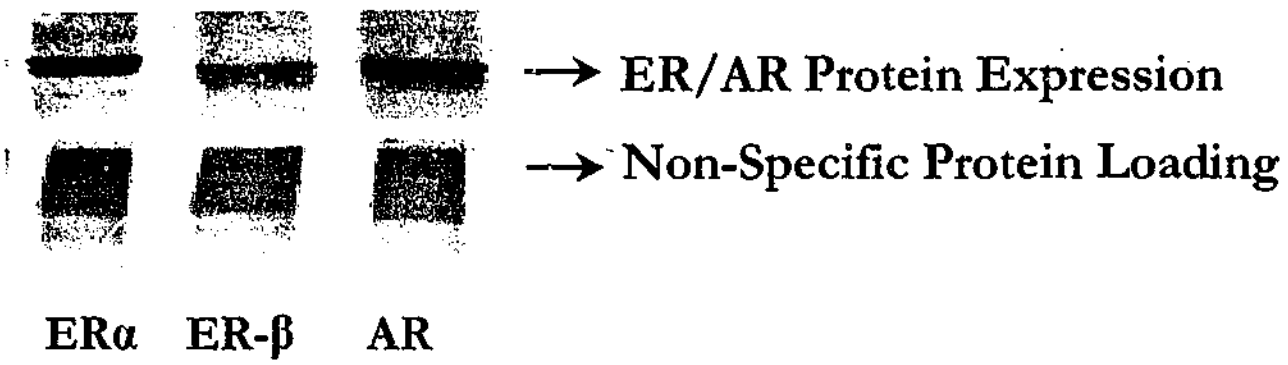
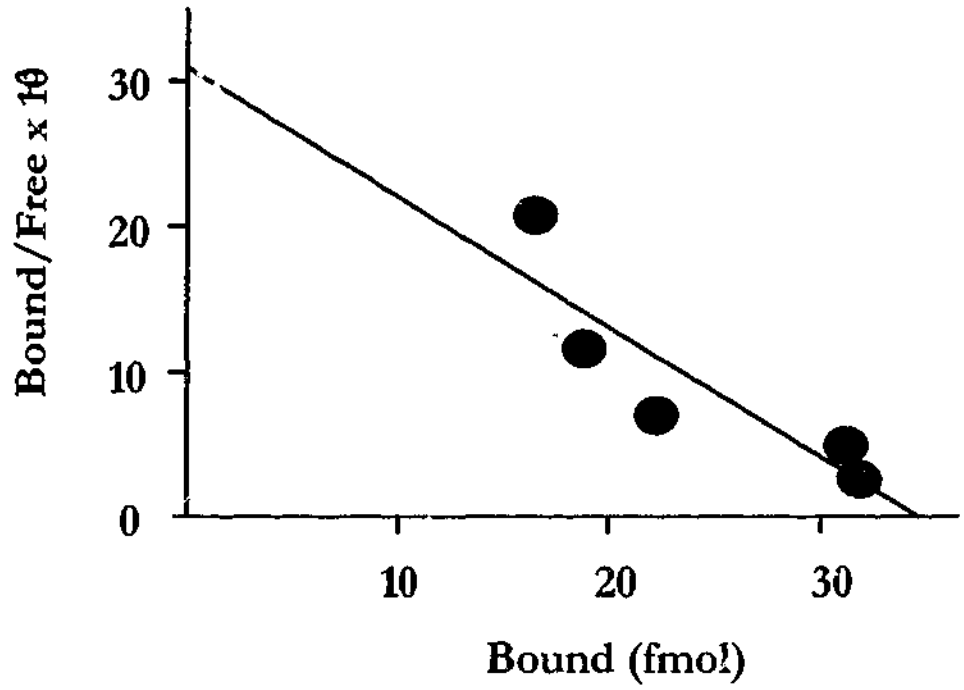


Figure 2B.1 Presence of ER and AR in VSMC

Western blot analysis confirmed the presence of ER-α, ER-β and AR in cultured VSMC



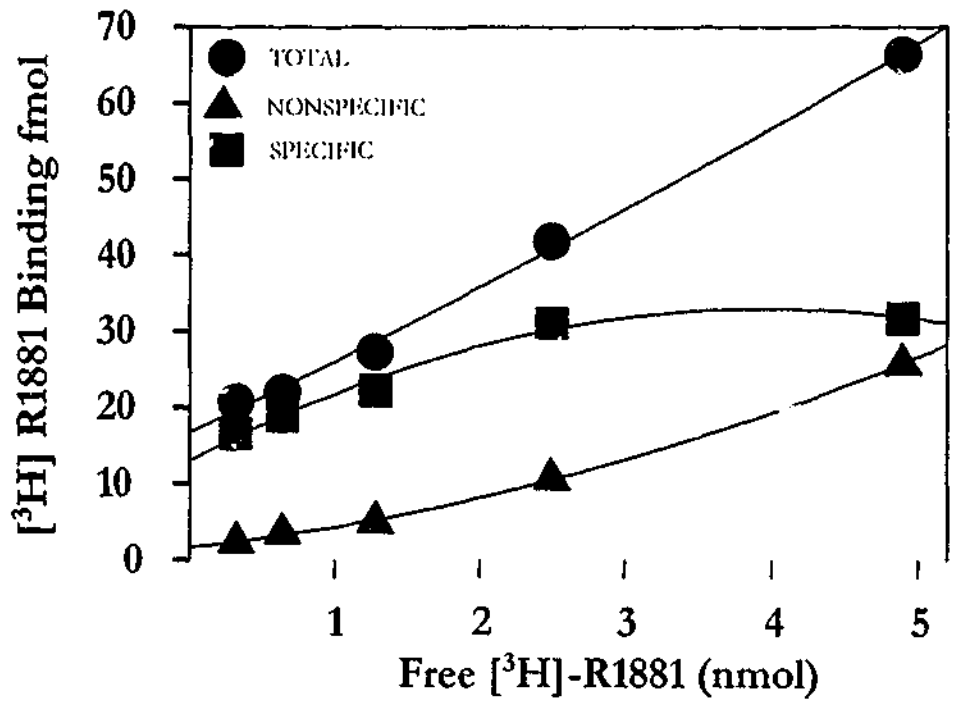
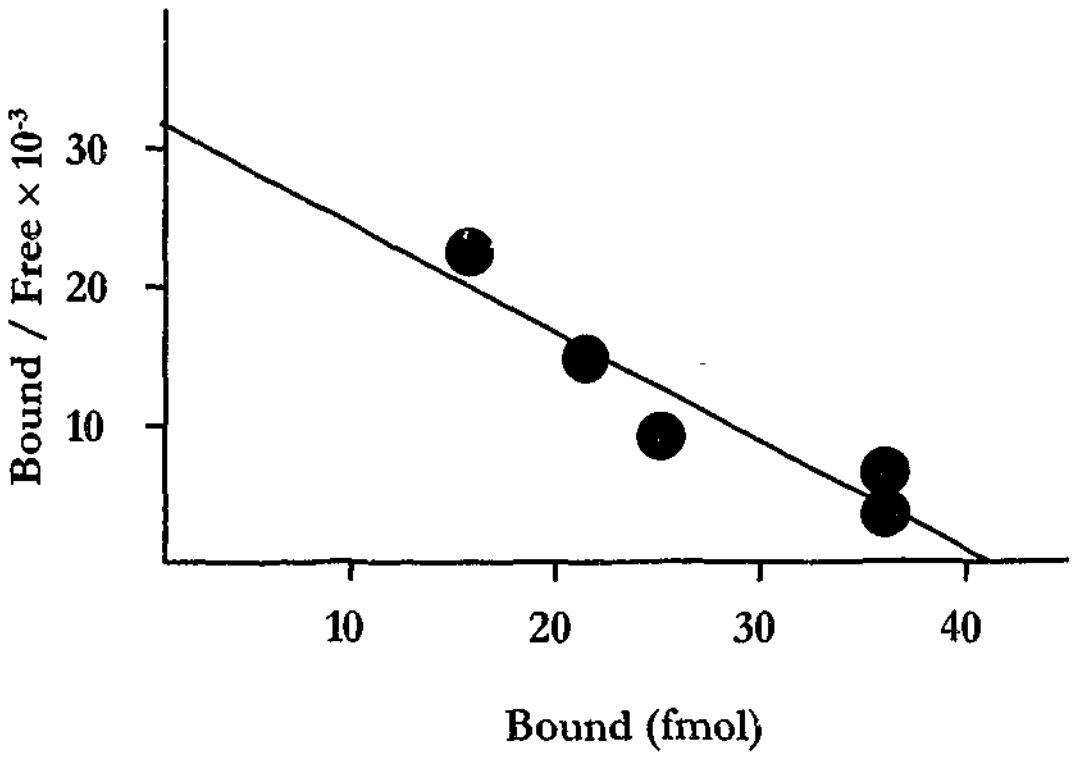


Figure 2B.2 Scatchard Analysis of Androgen Binding in in IMA VSMC

Scatchard analysis of [^{3}H]-R1881 (0.31-5 nmol/L) binding with and without 500-fold excess of nonradioactive dihydrotestosterone.



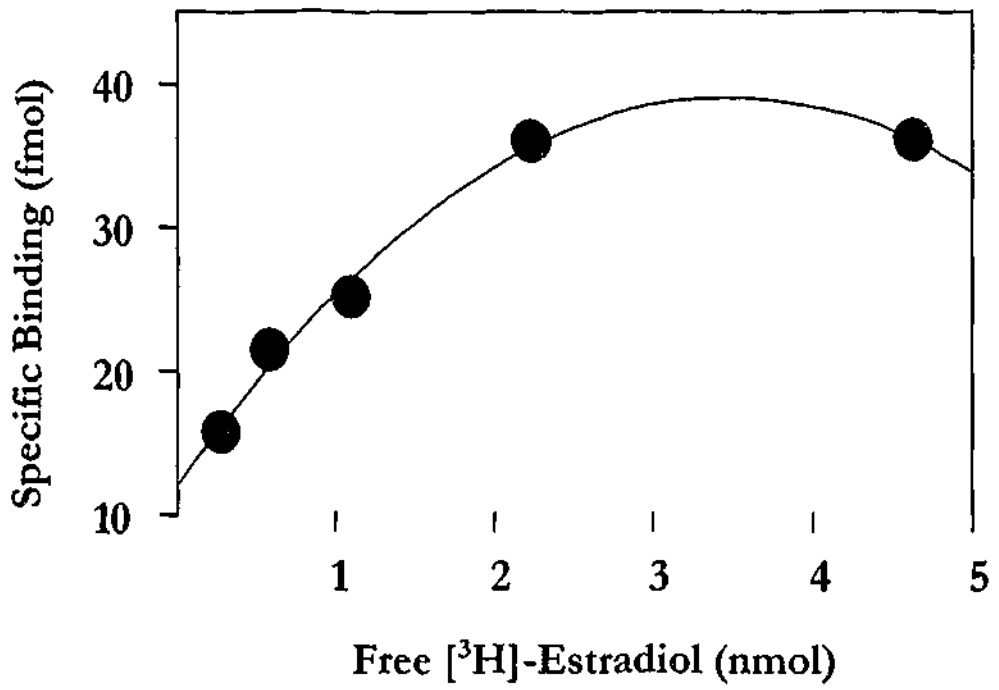


Figure 2B.3 Scatchard Analysis of Estrogen Binding in IMA VSMC

Scatchard analysis of [^{3}H]-17β-estradiol (0.315-5 nmol/L) binding with and without 500-fold excess of nonradioactive diethylstilbestrol.

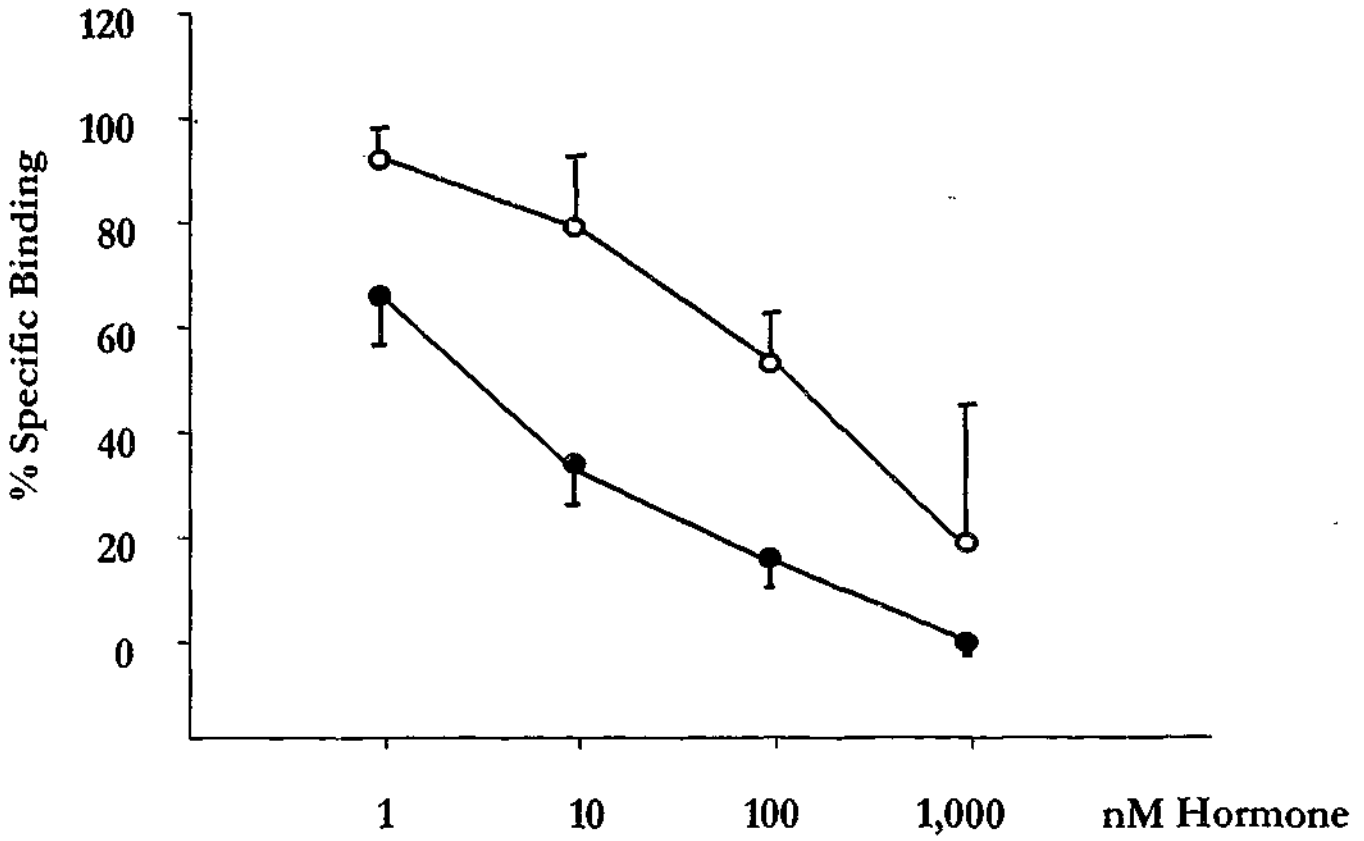


Figure 2B.4 DHEA Binding to AR in IMA VSMC

Displacement curves showing the presence of saturable binding sites for testosterone by the ability of nonradioactive testosterone to compete for binding with [3H]-R1881 (1nM) (—●—); non-specific binding (in the presence of 1 μM testosterone) varied from 60% to 75% of total. DHEA (1000 nM) (—○—) showed approximately 5% the potency of testosterone in binding to AR as shown by the displacement of 1 nM [^{3}H]R1881 (ie 20-fold higher DHEA concentrations are needed compared to testosterone).
n = 4, with 4 replicates in each experiment

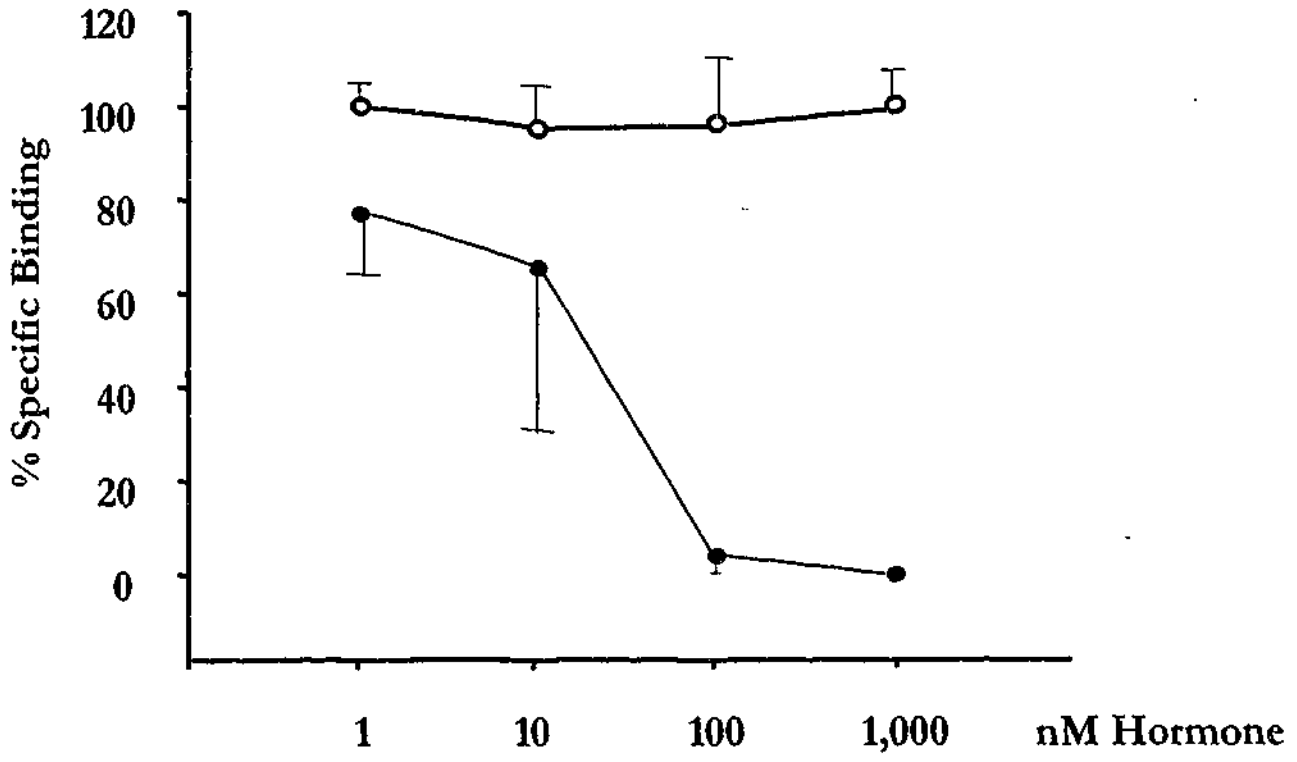
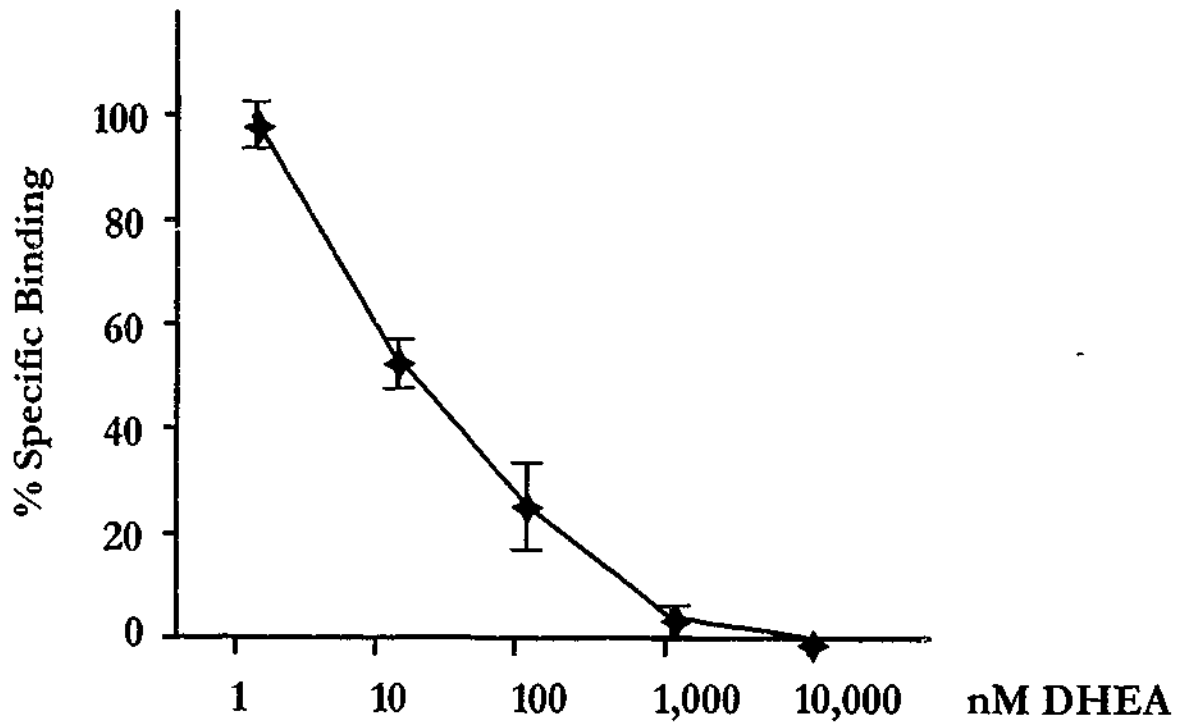


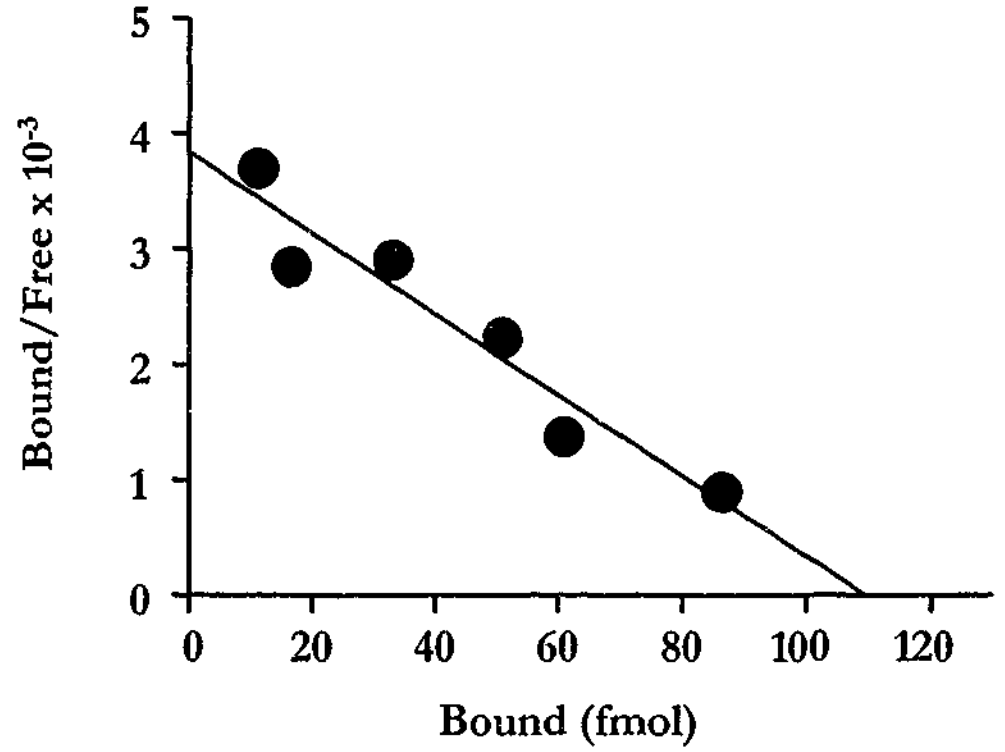
Figure 2B.5 DHEA Binding to ER in IMA VSMC

Displacement curves showing the presence of saturable binding sites for 17β-estradiol by the ability of nonradioactive 17β-estradiol to compete for binding with [3H]17β-estradiol (1nM) (—●—); non-specific binding (in the presence of 1 μM 17β-estradiol) varied from 50% to 65% of total. DHEA (1 – 1000 nM) (—○—) showed no specific binding to ER. n = 4, with 4 replicates in each experiment

A



B


Bound/Free x 10^{-3}
5
4
3
2
1
0
0
20
40
60
80
100
120
Bound (fmol)

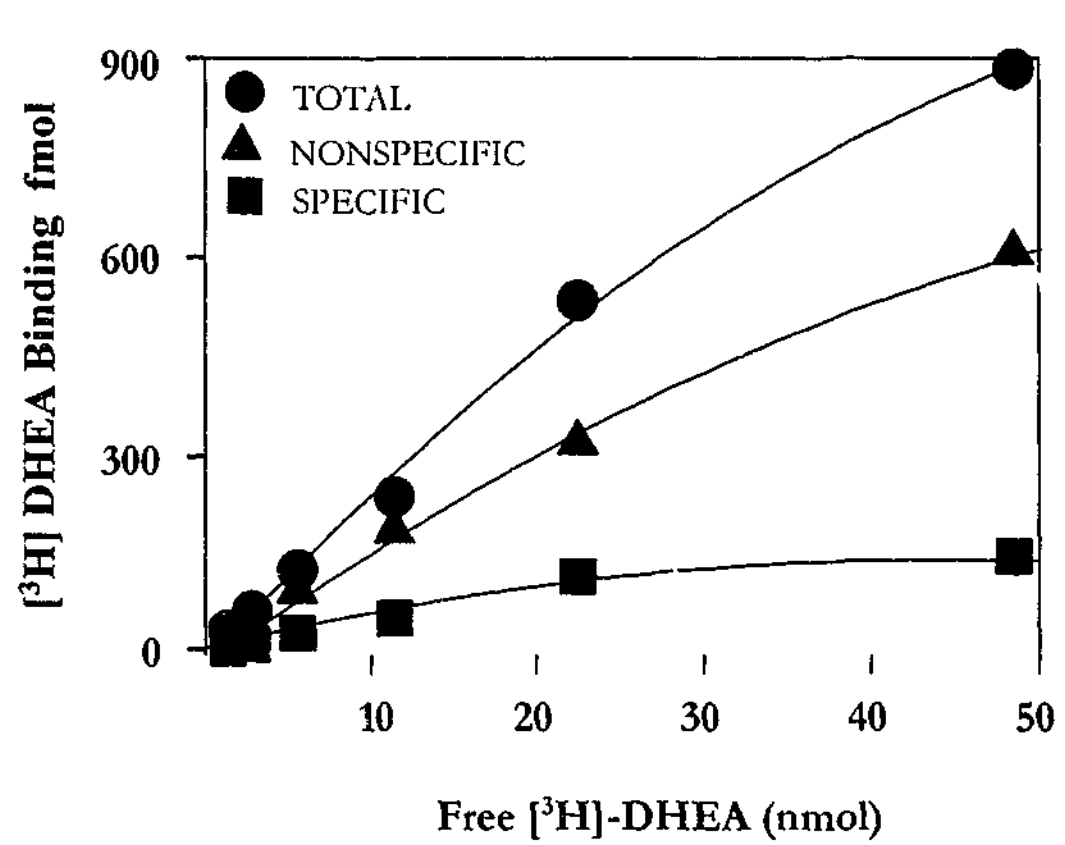

[^{3}H] DHEA Binding fmol
900
600
300
0
TOTAL
NONSPECIFIC
SPECIFIC
10
20
30
40
50
Free [^{3}H]-DHEA (nmol)

Figure 2B.6 Specific DHEA Binding in IMA VSMC

A. Displacement curve showing the presence of saturable binding sites for DHEA by the ability of nonradioactive DHEA to compete for binding with [3H]DHEA (10nM); non-specific binding (in the presence of 10 μM DHEA) varied from 50% to 65% of total. n = 6, with 4 replicates in each experiment

B. Scatchard analysis of specific [3H]DHEA (1.4-49nM) binding with and without 500-fold excess of nonradioactive DHEA.

2B.3 DISCUSSION

Despite being one of the most abundant steroid hormones in the circulation, the specific molecular and cellular mechanisms of DHEA action in the setting of cardiovascular physiology remain unclear. Three possible distinct pathways through which DHEA may exert physiological responses have been identified. First, DHEA may mediate a variety of functions by being enzymatically metabolized into androgens or estrogens (Milewich, 1983; Hayashi, 2000). Second, DHEA could also potentially act through binding to classical steroid hormone receptors, including ER (Poortman , 1977; Bruder , 1997; Kuiper, 1997) and AR (Sourla, 1998). Finally, DHEA could potentially act by binding to a specific DHEA receptor, and although putative binding sites have been demonstrated in murine T cells (Meikle, 1992) and rat liver (Kalimi, 1988), no such protein has been identified in vascular cells.

The results of this study indicate that a correlation exists between the DHEA-mediated inhibition of IMA VSMC proliferation (as described in Chapter 2A) and the presence of a binding site for this steroid hormone. As expected, in this study, testosterone and 17β-estradiol bound respectively to AR and ER in VSMC, with DHEA showing no binding to either receptor. DHEA did, however, exhibit specific binding to intact cells in a manner consistent with steroid hormone receptor-like interactions.

Although specific DHEA binding sites have previously been reported in rat liver and murine T cells, this is the first time a DHEA-specific binding site has been shown in VSMC. The affinity of this binding (Kd 37°C 14 nM), measured by saturation analysis

and shown in Fig. 2B.6 - B, is consistent with the circulating levels of DHEA *in vivo*. The high levels of nonspecific binding (70-85% of total, across the range of tracer concentrations used) make the value of 14 nM for the affinity an approximate one; the displacement of tracer down to 55% by an equal concentration (10nM) of non-radioactive DHEA is consistent with an affinity up to an order of magnitude lower (1-2 nM). Furthermore, it is unclear whether DHEA binds to other proteins such as sex hormone binding globulin (SHBG). Additional studies are required to address this question.

The results of this study together with those previously described (chapter 3A) suggest that DHEA influences VSMC behavior via direct DHEA-receptor complex interactions rather than by conversion to androgens or estrogens. However, whether biological responses to DHEA in other cell types and tissues are mediated by similar mechanisms is currently unknown. Further studies are required to investigate this hypothesis and to characterize the molecular structure of the putative DHEA receptor.

Part C

Sub-Cellular Mechanisms of DHEA Action – A Possible Role for Mitogen-Activated Protein (MAP)-Kinase

2C.1 METHODS

2C.1.1 Cell culture

IMA VSMC (10,000 cells/ml) were seeded in 60mm culture dishes and grown to near confluence in DMEM with 10% FBS. Following serum deprivation for 24 h, the cells were treated with DHEA (0.1 – 100 nM) for 4 hrs and then with PDGF-BB (10ng) for 15 min. Cells were then washed with ice-cold PBS, and scraped into ice-cold lysis buffer containing 0.25 M sucrose, 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM NaF, 0.1% 2-β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin A, 10 μM leupeptin, and 0.23 U/ml aprotinin. After centrifugation at 100,000 g for 10 min at 4°C the supernatant was taken for MAP kinase activity assay, and cell lysate protein levels measured by the Bradford method. For positive controls in the JNK and p38 assay systems, IMA VSMCs were treated with TNFα (20ng) for 10 min or ultraviolet light (15 sec) prior to isolating cystolic protein fractions.

2C.1.2 ERK-1, JNK and p38 protein kinase assays

Kinase assays for ERK-1, JNK and p38 kinases have been previously described (Li 1997). Briefly, 50 µg of cytosolic protein was incubated (4 ^{0}C for 1 hour) in lysis buffer containing 50 mg/mL BSA and specific anti-ERK1, anti-JNK or anti-p38 antibodies. This was followed by a second incubation (4 ^{0}C for 1 hour) with protein A-sepharose beads and a third incubation (30^{0}C for 15 min) with 40 µL of protein kinase buffer containing 20 mM Hepes (pH 7.6), 10 mM $MgCl_2$, 10 mM 2-β-glycerophosphate, 0.1 mM Na-vanadate, 2 mM DTT, 0.1 µM microcystin, 5 µg/mL PKI, 50 µM ATP, 3 µCi/tube [γ-^{32}P]-ATP and specific substrate proteins for the kinase type; 0.25 mg/mL MBP for ERK1 kinase, 0.3 mg/mL c-Jun (5-79)-GST fusion protein for JNK kinase, and ATF-2 GST fusion protein for p38 kinase. Following termination of the reaction by the addition of 20 µL of SDS buffer and cooling to 4°C, samples (20 µL) were subjected to electrophoresis on 15% SDS/polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane and the membrane was then autoradiographed to show kinase phosphorylation. Phosphorylation was quantified by scanning the autoradiographed bands and measuring band pixel density using imaging software. To verify the immunoreactivity of the kinases, Western blotting was carried out on the nitrocellulose membrane using the same antibodies as for immunoprecipitation as described above.

2C.1.3 Statistical analysis

All data are presented as mean ± SEM. Statistical analysis between two observations was by Student's t-test and in multiple comparisons by analysis of variance. The null hypothesis was rejected at $p>0.05$.

2C.2 RESULTS

2C.2.1 Effects of DHEA on mitogen activated protein kinase (MAP kinase) activity in the presence of PDGF-BB or TGF-β1

DHEA (10^{-10} – 10^{-7} M) inhibited PDGF-BB induced increases in ERK1 kinase activity, as measured by MBP phosphorlyation, in a dose dependent manner (Fig 2C.1). Neither JNK nor p38 kinase phosphorylation was influenced by either PDGF-BB or DHEA, although both were appropriately stimulated by TNFα and ultraviolet light in human VSMC (Fig 2C.2).

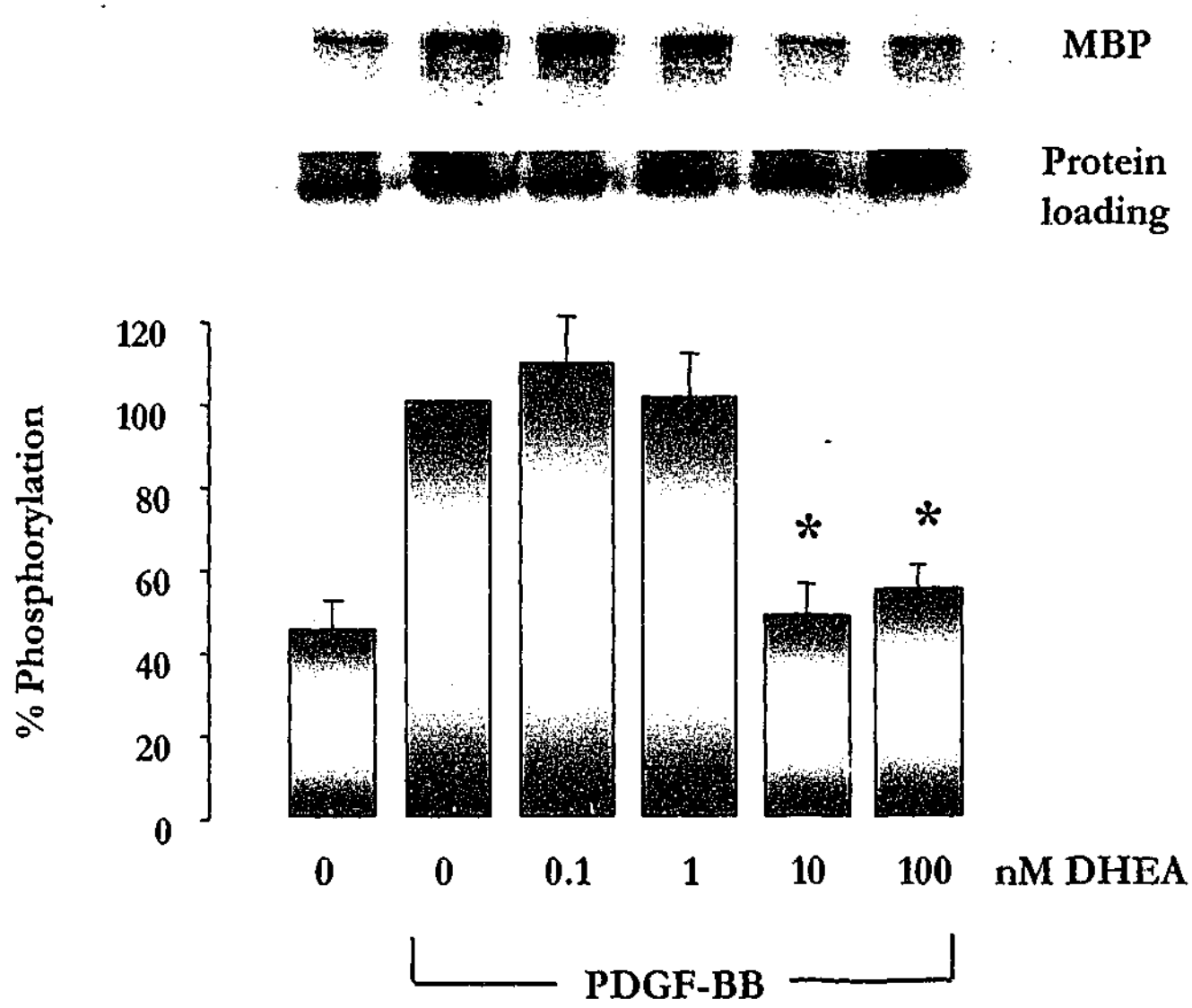


Figure 2C.1 The effects of DHEA on ERK1 kinase phosphorylation
DHEA (0.1 – 100 nM) inhibits PDGF-BB induced increases in ERK1 kinase activity in a dose dependent manner.

Data are presented as mean percentage of PDGF-BB stimulation of ERK1 phosphorylation ± SEM.
* denotes a significant difference from PDGF-BB induced ERK1 phosphorylation in VSMC.
n =4

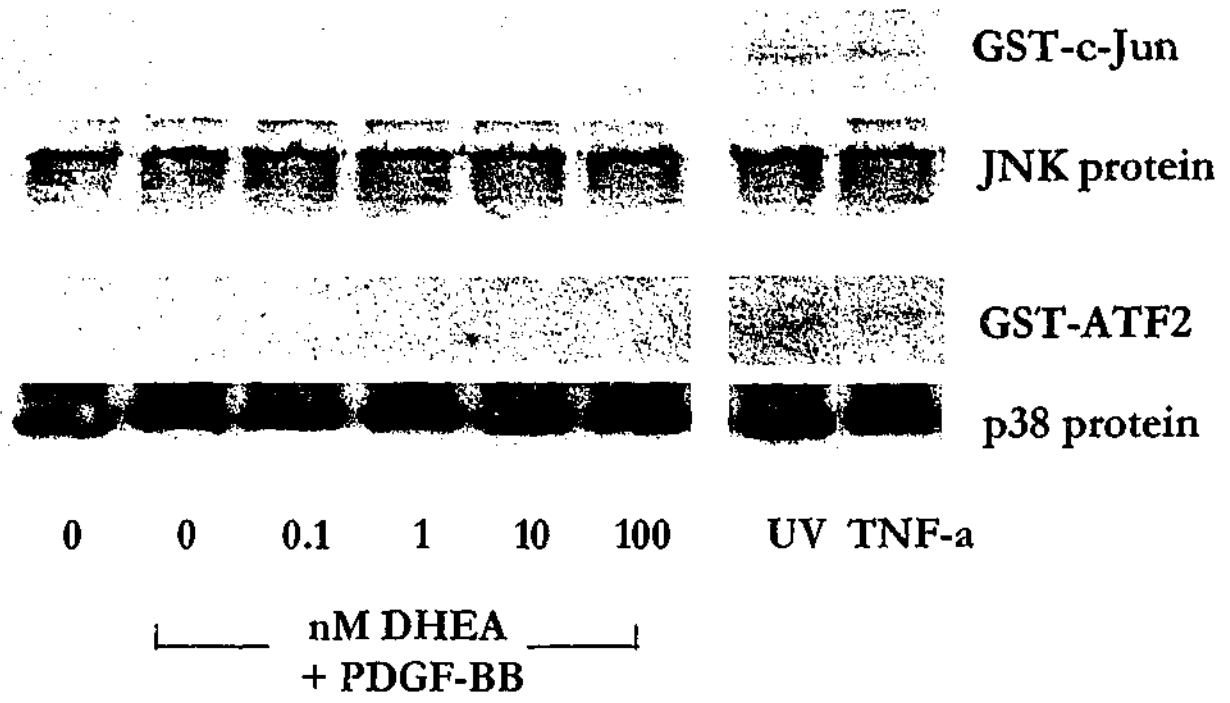


Figure 2C.2 The effects of DHEA on JNK and p38 kinase phosphorylation

Neither JNK nor p38 kinases are not phosphorylated in quiescent VSMC and neither is influenced by PDGF-BB or DHEA, although both are appropriately stimulated by TNFα and ultraviolet light in human VSMC.

n = 5

2C.3 DISCUSSION

Mitogen-activated protein (MAP) kinase consists of a superfamily of protein kinases including extracellular signal-regulated kinase (ERK), c-jun N-terminal protein kinase (JNK) and reactivating proteing kinase, RK (p38) (Zhu, 1999). MAP kinases are major components of pathways controlling cell differentiation, proliferation and death (Pearson, 2001). To investigate the possible subcellular mechanisms through which DHEA may exert its effects on VSMC proliferation we sought to examine subcellular mechanisms involving mitogen-activated protein kinase (MAP-kinase), which has been implicated in other steroid responses, including those induced by estrogens and androgens (Kalimi, 1988; Miyamoto, 1998; Goodenough, 2000; Singh, 2000).

While estrogens have been shown to inhibit VSMC proliferation via MAP kinase signaling pathways (Morey, 1997), it is not known whether androgens affect VSMC proliferation via similar mechanisms. A previous study has demonstrated that DHEA inhibited PDGF-BB induced MAP kinase phosphorylation in a dose dependent manner (Singh, 2000), suggesting a potential subcellular mechanism of DHEA action via the MAP kinase pathway. However, the authors of this report did not differentiate between the different kinases that constitute the MAP kinase superfamily.

The present study investigated the effects of DHEA on PDGF-BB induced ERK-1, JNK and p38 phosphorylation. It has been demonstrated that DHEA significantly inhibits PDGF-BB induced ERK-1 phosphorylation in a dose dependent manner. JNK and p38 kinase protein were shown to be present in high levels but not phosphorylated

in quiescent human VSMC; neither PDGF-BB nor DHEA affected JNK or p38 kinase levels or phosphorylation status. However, both JNK and p38 are phosphorylated by TNFα and ultraviolet light (Fig. 2C.2). We therefore suggest that endogenous JNK and p38 kinases are inactive in human quiescent VSMC, and that the observed DHEA effects are mediated specifically via ERK-1 signaling pathways.

2.2 CONCLUSIONS

This series of experiments has investigated the effects of DHEA on the proliferation of human vascular smooth muscle cells and has provided direct evidence that the hormone attenuates human vascular smooth muscle cell proliferation by mechanisms independent of either androgen or estrogen receptors. These effects may be attributable to interactions between DHEA and DHEA-specific binding complexes and may be mediated, at least in part, by ERK-1 signaling pathways. The actions of DHEA seen are potentially beneficial in the setting of cardiovascular pathophysiology.

Chapter 3

DHEA Action in Endothelial Cells:

Proliferation, Receptors & Regulatory Pathways

3.1 INTRODUCTION

The endothelium is a dynamic organ that lines the interface between the blood and artery walls. It has the capacity to respond rapidly to an array of agonists and varying environmental conditions by elaborating a number of proteins, prostanoids and other paracrine substances to preserve cardiovascular homeostasis. The endothelium plays an integral role in the maintenance of vascular integrity by its participation in thrombolytic control, secretion of cytokines, platelet and leukocyte interactions with the vessel wall, regulation of vasomotor tone, and cellular growth (as detailed in Chapter 1.3).

Despite its many structural and regulatory functions, the vascular endothelium represents a unique cell population, in that endothelial cells only grow as a strict monolayer. In addition, under normal physiological conditions, endothelial cell angiogenesis is virtually absent, and cell turnover rate is significantly lower than that of other cell types (Fallon, 1972). However, in states such as diabetes and atherosclerosis, the anatomical and functional integrity of the vascular endothelium is compromised (Ross, 1993), leading to the exacerbation of thrombosis, vasoconstriction and increased VSMC proliferation. Therefore, increased endothelial cell migration and proliferation at the site of injury is required for vessel recovery and prevention of further CVD progression (Schwartz, 1981).

Estrogens have been claimed to confer cardioprotective effects in premenopausal women and in postmenopausal women taking hormone-containing therapies. Accordingly, much interest has been focused on the impact of estrogens on

atherosclerotic CVD progression via effects on the structural and functional integrity of the vascular endothelium. Indeed, estrogens have been shown to augment endothelial cell migration and proliferation (Morales, 1995; White, 1997; Somjen, 1998; Suzuma, 1999), by classical ER mediated mechanisms (Xiao, 1999). The subcellular mechanisms by which estrogens influence endothelial cell proliferation include the up-regulation of vascular endothelial growth factor (VEGF) receptors (Suzuma, 1999), activation of ERK-1/2 and p38 MAP-kinase activity (Razandi, 2000) and inhibition of endothelial cell apoptosis (Razandi, 2000).

NO is also considered to play an integral role in endothelial cell angiogenesis (in addition to regulating vasomotor tone), although experimental results are conflicting. Reports have suggested that NO inhibits endothelial cell migration (Lau, 1996) and proliferation (Pipili-Synetos, 1993; Pipili-Synetos, 1994; Heller, 1999). Other studies, however, have shown that NO is essential for endothelial cell angiogenesis (Papapetropoulos, 1997; Murohara, 1998; Parenti, 1998). A study by Gooch et al. (1997) suggested that relatively large concentrations of exogenous NO inhibit endothelial cell proliferation, whereas endogenous levels of NO are inadequate to inhibit proliferation (Gooch, 1997).

Physiological concentrations of estrogen have been shown to stimulate NO release from endothelial cells through the activation of ER (Stefano, 2000). In addition, it has been reported that estrogen protects against vascular injury by stimulating endothelial cell migration and proliferation by mechanisms involving the up-regulation of plasma NO (White, 1997). The authors of this study concluded that increases in NO production may be a fundamental mechanism by which estrogen blunts vascular injury.

The role of estrogens in modulating the integrity of the vascular endothelium via profound effects on endothelial cell migration and proliferation has been extensively studied. In contrast, the role of androgens on similar parameters of vascular function has attracted little attention. There is, however, limited evidence suggesting that androgens, particularly dihydrotestosterone, influence endothelial function *in vitro* by promoting cell proliferation (Somjen, 1998). *In vivo* studies investigating the effects of androgens on endothelial function have yielded conflicting results. A number of reports have suggested that testosterone enhances NO-mediated endothelium-dependent vasodilation in men (Chou, 1996; Ong, 2000). More recently, however, testosterone has been shown to reduce arterial diameter and increase vascular tone (Geary, 2000), a potentially deleterious effect.

In considering the current evidence in support of a beneficial influence of DHEA in cardiovascular physiology (Chapter 1.6), a potentially important avenue of DHEA-mediated effects is via direct influences on endothelial cell behavior. However, following a comprehensive review of the literature, only two studies relevant to this field of research have been identified. The first is an early study by Sholley et al. (1990), showing that DHEA accentuates the formation of multimellar lipid structures, identified as lysosomes, in cultured human endothelial cells (Sholley, 1990). Furthermore, DHEA has been shown to significantly inhibit the propagation of vascular endothelial cells in culture (Mohan, 1997), an effect opposite to those of estrogens and androgens previously reported.

Both of these studies suggest that DHEA may be potentially harmful in the settings of atherogenesis and general cardiovascular health. However, inherent difficulties arise when attempting to extrapolate the physiological relevance of the observed DHEA effects. The first impediment is the obvious deficiency in the existence of comprehensive scientific evidence regarding the effects of DHEA on endothelial cell behavior. The second difficulty, as highlighted in Chapter 2, is the issue of DHEA conversion to other androgenic and estrogenic metabolites, which has been shown to occur in vascular endothelial cells (Milewich, 1983). In addition, the possibility of specific DHEA receptors being present on vascular endothelial cells and the subcellular regulatory pathways by which DHEA might exert its vascular effects, are not known.

Aims of Study

To address the issue of the potential effects of DHEA on endothelial cell behavior This study sought to examine the actions of DHEA on the proliferation of cultured endothelial cells and on the potential regulatory pathways through which these effects might be mediated, including MAP-kinase signaling pathways and eNOS synthesis.

Further, to investigate whether the effects of DHEA on endothelial cell proliferation are mediated via DHEA conversion to androgens or estrogens, or by direct DHEA action, the effects of DHEA on endothelial cell proliferation in the presence of AR and ER antagonists, was examined. DHEA binding studies were also performed to investigate the possible existence of specific DHEA receptor in these cells.

Part A

DHEA Increases Endothelial Cell Proliferation Independently of Androgen and Estrogen Receptors

3A.1 METHODS

3A.1.1 Experimental Protocol

Bovine aortic endothelial cells (BAEC)

BAEC were kindly donated by Dr. Peter J. Little of the Baker Medical Research Institute, Melbourne, Australia. BAEC were isolated by enzymatic digestion from the aortas of young calves. Briefly, aortas were obtained aseptically at abattoirs and transferred to the laboratory; under sterile conditions the aorta was transected longitudinally. A sharp blade was passed gently over the luminal surface and rinsed into collagenase (3 mg/ml, Worthington, N.J.). The cell suspension was incubated for 5 minutes at 37°C, diluted with RPMI containing 20% fetal bovine serum and centrifuged (900 rpm) for 5 minutes. The cell pellet was resuspended in the same media and incubated at 37°C in an atmosphere of 5% carbon dioxide in air. The media were changed every 3 days and after several weeks the cells proliferated to form a monolayer which microscopically appeared as a cobblestone network of cells. The cells were passaged routinely for several weeks and characterized immunohistochemically in early

and late passages and both cases by positive staining for von Wilebrand factor and negative staining for smooth muscle α-actin.

3A.1.2 Cell proliferation

BAEC proliferation was determined by directly counting cell numbers. Cells were seeded in to 24-well plates at a density of 5,000 cells/mL, grown to 60-70% confluence, treated with either DHEA, 17β-estradiol or testosterone (1 – 100nM) and FBS (2.5%) as described in chapter 2A.1.2. After 48 h, cells were harvested and counted in an automatic cell counter (S.ST.H/ZM, Coulter Electronics Ltd., England).

3A.1.3 Pharmacological antagonism of ER and AR

To determine whether the effects of DHEA on cell proliferation are mediated via ER or AR, measurements of cell proliferation were performed in the presence and absence of the ER antagonist ICI 182,780 or the AR antagonist flutamide added 2 h prior to the addition of the various hormones as described in chapter 2A.1.3.

3A.1.4 Statistical analysis

All data are presented as mean ± the SEM. Statistical analysis between two observations was by Student's t-test and in multiple comparisons by analysis of variance. The null hypothesis was rejected at $p>0.05$.

3A.2 RESULTS

3A.2.1 Effects of DHEA on endothelial cell proliferation

DHEA, 17β-estradiol, and testosterone (0.1-100 nmol/L) accentuated FBS-induced increases in cell proliferation in BAEC in a dose dependent manner, with a maximum effect of DHEA to 138±3%, 17β-estradiol to 119±4% and testosterone 127±4% of control (Fig 3A.1). These results demonstrate that DHEA increases FBS-induced BAEC proliferation in a fashion similar to estrogen and testosterone.

3A.2.2 Effects of AR and ER antagonists

The stimulatory effect of DHEA (10 nmol/L) on FBS induced cell proliferation was not affected by either flutamide or ICI 182,780 (100 nmol/L). However, flutamide completely abolished the stimulatory effects of testosterone, and ICI 182,780 blocked the effects of 17β-estradiol (Fig 3A.2). This provides evidence that the actions of DHEA are not mediated by either AR or ER. Flutamide and ICI 182 780, alone or combined, had no effects on cell proliferation.

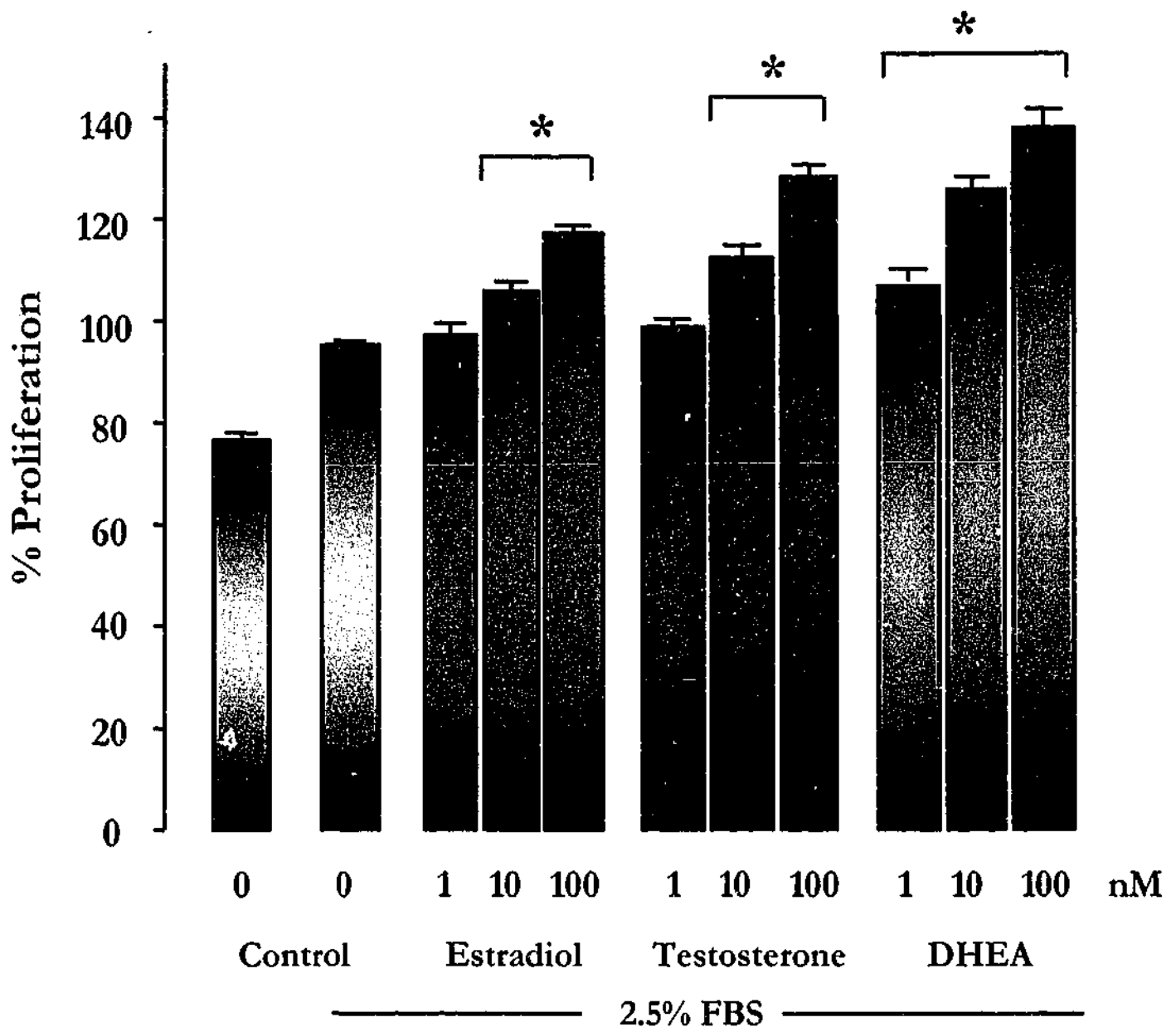


Figure 3A.1 The Effects of Sex Hormones on Endothelial Cell Proliferation
DHEA, 17β-estradiol and testosterone (1 – 100 nM) increase FBS-induced increases in endothelial cell proliferation in a dose dependent manner, with a maximum stimulatory effect of DHEA to 138±3% compared with 17β-estradiol and testosterone (119±4% and 127±9% of control respectively).

Data are presented as mean percentage of FBS stimulation of cell proliferation ± the standard error of the means (SEM).
* denotes a significant difference from FBS-induced increases endothelial cell proliferation.
n = 6, with 4 replicates in each experiment

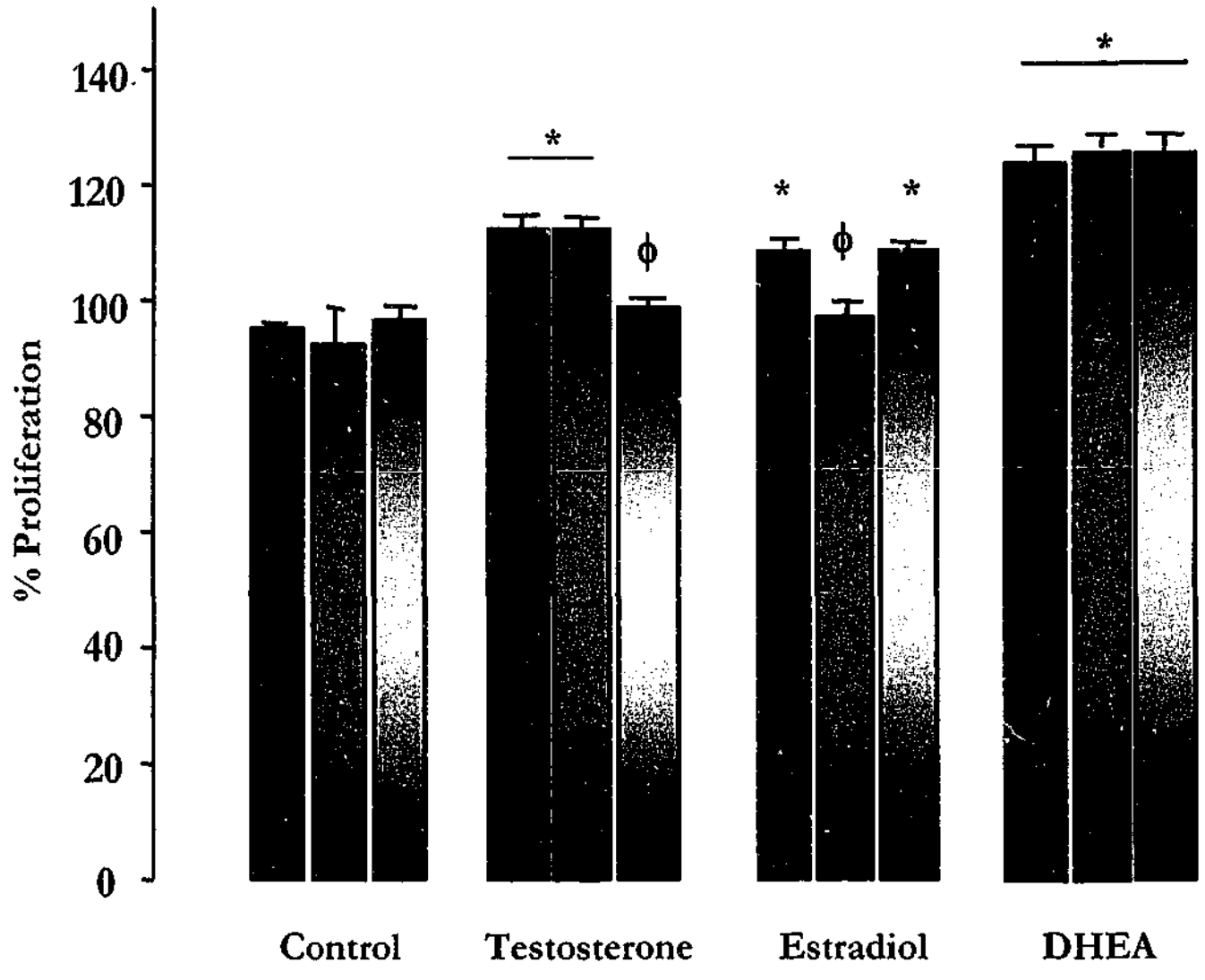


Figure 3A.2 The Effects of the AR antagonist flutamide and the ER antagonist ICI 182,780 on DHEA-induced stimulation of endothelial cell proliferation

The AR antagonist flutamide (100 nmol/L) completely abolishes the stimulatory effects of testosterone (10 nmol/L), and the ER antagonist ICI 182,780 blocks the effects of 17β-estradiol, but neither antagonist affects the stimulatory actions of DHEA on endothelial cell proliferation. Cells were pretreated with AR or ER antagonists (100 nmol/L, 2h) prior to exposure to steroids (10 nmol/L).

Data are presented as mean percentage of FBS stimulation of cell proliferation ± SEM.

* denotes a significant difference from FBS- induced increases in cellproliferation

ϕ denotes a significant difference from the hormone-alone treatment.

n = 4, with 4 replicates in each experiment

Hormone alone

Hormone + ICI 182 780

Hormone + Flutamide

3A.3 DISCUSSION

There is a substantial body of evidence highlighting the importance of sustained endothelial integrity in the regulation of vascular function in health and disease. In addition estrogens, and to a lesser extent androgens, have been shown to contribute to the maintenance of vascular function via direct effects on the vascular endothelium. This is, however, the first study to provide direct evidence that DHEA stimulates vascular endothelial cell proliferation by mechanisms independent of either androgen or estrogen receptors.

Consistent with previous reports, 17β-estradiol stimulated endothelial cell proliferation via classical ER-dependent pathways. In addition, we have shown, for the first time, that testosterone also has similar stimulatory effects. The AR antagonist flutamide effectively blocked the effects of testosterone, while exerting no effect on its own. Neither flutamide nor ICI 182 780 influenced the stimulatory effects of DHEA on endothelial cell proliferation, indicating that the actions of DHEA seen are independent of AR and ER.

To our knowledge, this is the first study showing that testosterone accentuates endothelial cell proliferation in a manner that closely resembles the action of estrogens. The observation that the AR antagonist flutamide abolishes the stimulatory effects of testosterone illustrates the specificity of the observed androgenic effects. These findings are supported by those of a previous report by Somjen et al. (1998), demonstrating stimulatory effects of dihydrotestosterone on endothelial cell proliferation and inhibitory

effects on VSMC proliferation by AR-mediated pathways. While these observations suggest a potentially beneficial role for androgens in vascular wall remodeling, they are not supported by our previous report, and that of others (Fujimoto, 1994), that testosterone increases VSMC proliferation (Chapter 3A), a potentially deleterious effect. The opposing effects of testosterone on endothelial cell and VSMC proliferation and their physiological relevance are beyond the scope of this report and warrant further investigation.

The effects of DHEA on cellular angiogenesis are consistent with the concept that DHEA might confer beneficial effects on vascular function by increasing the rate of reendothelialization and inhibiting VSMC proliferation (as demonstrated in chapter 3A). It is known that the disruption of the anatomical and functional integrity of the endothelium and the unopposed proliferation of VSMC are hallmark features of atherosclerotic disease. Accordingly, the opposing effects of DHEA on endothelial cell and VSMC proliferation provides a potential mechanism for the postulated atheroprotective effects of DHEA.

The specificity of DHEA action in stimulating endothelial cell proliferation is supported by a number of observations. In the first instance, the present study has demonstrated that the effects of DHEA on endothelial cell proliferation are independent of both AR and ER, evidenced by the DHEA induced response being unaffected by either flutamide or ICI 182,780. In contrast, the effects of 17β-estradiol and testosterone were completely abolished by their respective receptor antagonists. We have also reported similar interactions between DHEA and VSMC, in that DHEA inhibits cell proliferation by

mechanisms independent of AR and ER (Chapter 2A). The identification of specific DHEA-binding sites in VSMC (Chapter 2B) lends further support for a direct effect of DHEA on the behavior of the individual components comprising the vascular wall, specifically VSMC and endothelial cells. Further studies are required to assertain the possible physiological implications of these findings.

Part B

DHEA-Specific Binding Sites in Vascular Endothelial Cells

3B.1 METHODS

3B.1.1 ER and AR studies

ER and AR density in BAEC was determined by radioligand binding assays as described previously (Chapter 2B.1.2). Cells were grown to confluence and incubated with [^{3}H]-estradiol (0.315-5 nmol/L) with or without non-radioactive diethylstilbestrol (DES, 1 μmol/L) for ER, and [^{3}H]-R1881 (0.31-5 nmol/L) with 1 μmol/L TA plus or minus nonradioactive DHT (1 μmol/L) for AR, for 90 min. Cells were scraped out and extracts put into scintillation vials for counting in a β-counter. ER and AR protein expression was analyzed by Western blot (as described in Chapter 2B1.1). The radioligand binding assays were kindly carried out by Dr Shanhong Ling.

3B.1.2 ER and AR binding assays

Competitive binding assays were carried out to determine the relative binding affinity of DHEA for ER and AR in BAEC, as described in Chapter 2B.1.2. In brief, confluent monolayers of BAEC were treated with DHEA (1-10,000 nmol/L) plus 1 nmol/L of either [^{3}H]-estradiol or [^{3}H]-R1881, with testosterone or 17β-estradiol as controls.

Following incubation at 37 °C for 60 min, cells were washed with D-PBS, treated with 0.1% TCA and harvested. Extracts were transferred to scintillation vials for counting in a β-counter.

3B.1.3 DHEA binding assays

Competitive binding assays were carried out to explore DHEA binding to intact cells as described in Chapter 2B.1.3. Confluent monolayers of BAEC were treated with [^{3}H]-DHEA (10 nmol/L) and 1-10,000 nmol/L non-radioactive DHEA (90 min). Cells were harvested and transferred to scintillation vials for counting in a β-counter.

Scatchard analysis was carried out to determine the affinity of DHEA binding. Confluent monolayers of BAEC were treated with [^{3}H]-DHEA (49-1.5 nmol/L) in the presence or absence of 10 000 nmol/L non-radioactive DHEA (90 min). Cells were harvested and transferred to scintillation vials for counting in a β-counter.

3B.1.4 Statistical analysis

All data are presented as mean ± SEM. Statistical analysis between two observations was by Student's t-test and in multiple comparisons by analysis of variance. The null hypothesis was rejected at $p>0.05$.

3B.2 RESULTS

3B.2.1 Presence of AR and ER

While Western blot analysis confirmed the expression of both AR and ER protein in BAEC (Fig 3B.1), radioligand assays demonstrated the presence of AR (at the density of ~24,000 sites/cell, Kd = 12 nmol/L) (Fig 3B.2) but not ER in the cells (Fig 3B.3).

3B.2.2 DHEA Binding to AR and ER

DHEA at concentrations of 10,000 nmol/L did not displace [^{3}H]R1881 binding to AR (Fig 3B.4). As 17β-estradiol did not show any specific binding, no competitive DHEA binding for ER was carried out.

3B.2.3 Specific DHEA Binding

The presence of saturable binding sites for DHEA was demonstrated by the ability of nonradioactive DHEA to compete for binding with [^{3}H]DHEA (10nM); non-specific binding (in the presence of 1 μM DHEA) varied from 70% to 80% of total (Fig 3B.5a). Scatchard analysis of specific [^{3}H]DHEA (0.28-61nM) binding with and without 500-fold excess of non-radioactive DHEA showed that there were ~33,600 binding sites/cell, binding [^{3}H]DHEA with an affinity given by Kd 37°C 19nM (Fig 3B.5b).

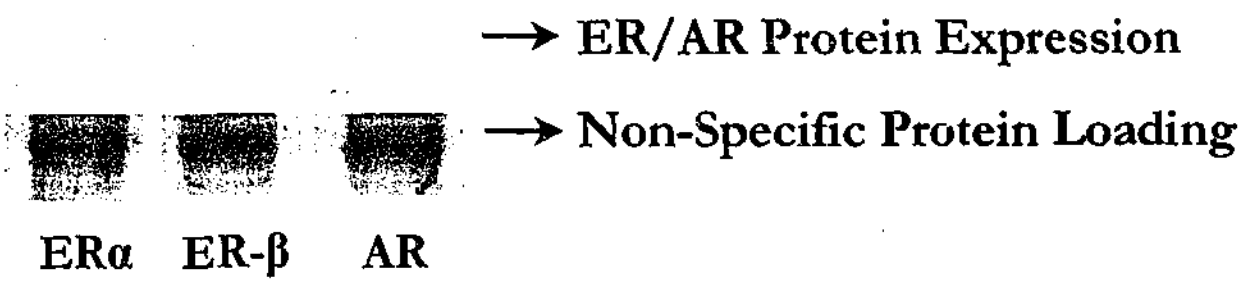
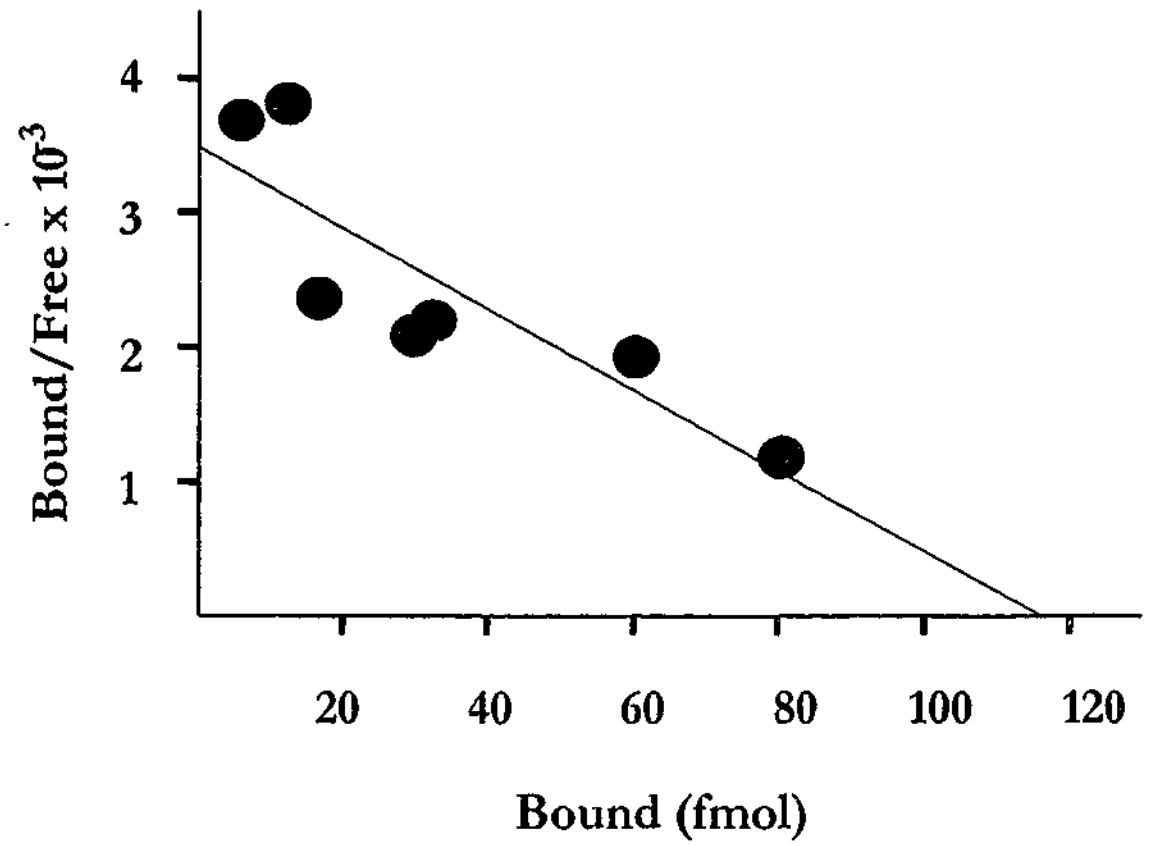


Figure 3B.1 Presence of ER and AR in Endothelial Cells

Western blot analysis confirmed the presence of ER-α, ER-β and AR in cultured endothelial cells.



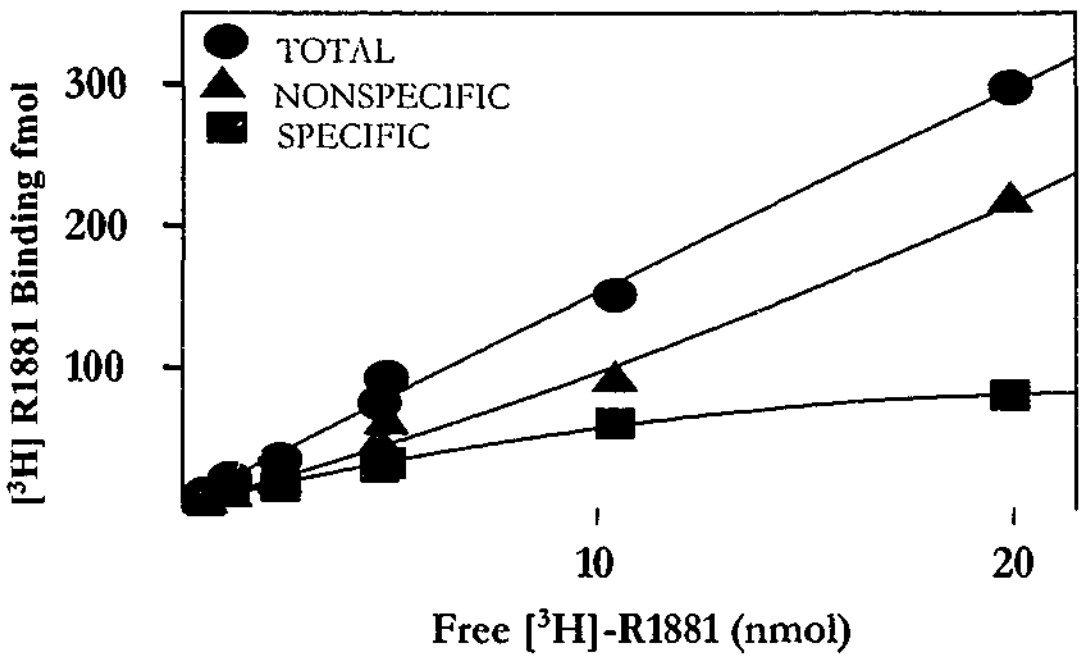
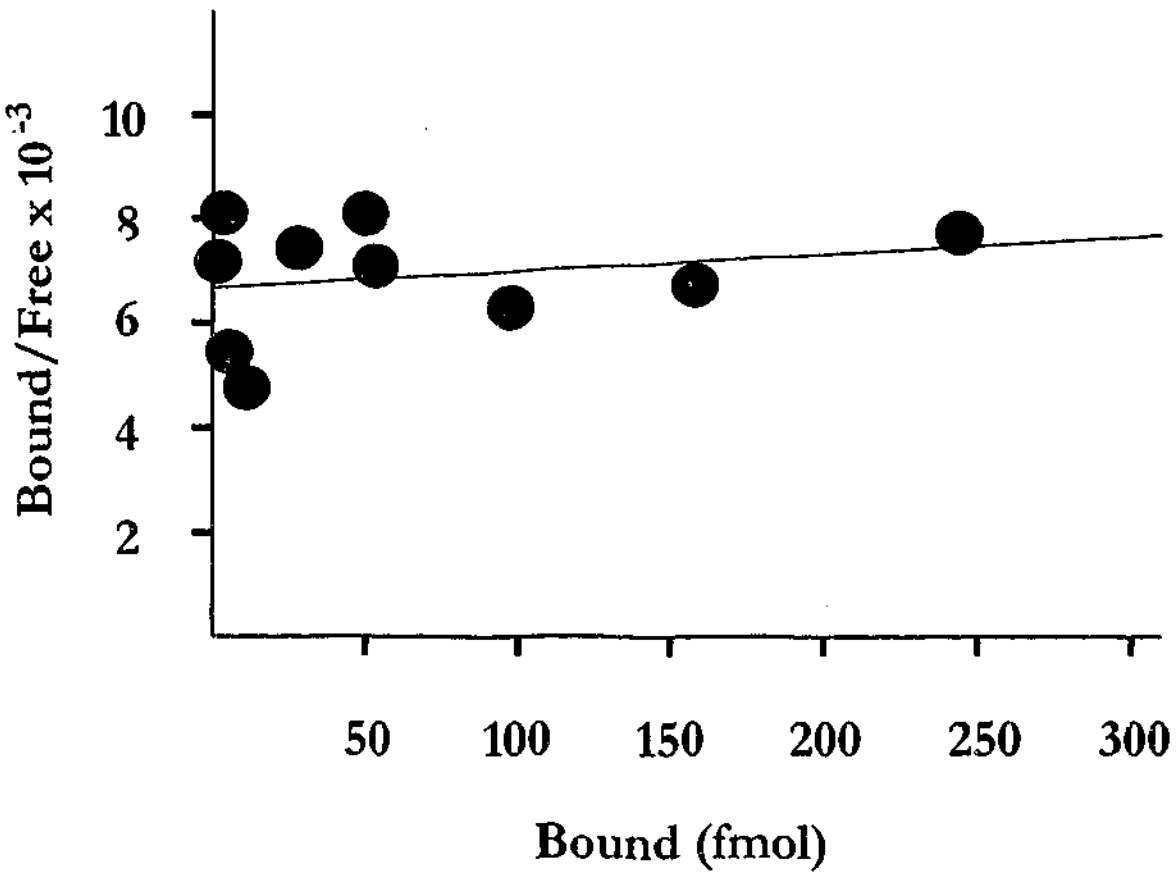


Figure 3B.2 Scatchard Analysis of Androgen Binding in in Endothelial Cells

Scatchard analysis of [^{3}H]-R1881 (0.31-5 nmol/L) binding with and without 500-fold excess of nonradioactive dihydrotestosterone

n = 3



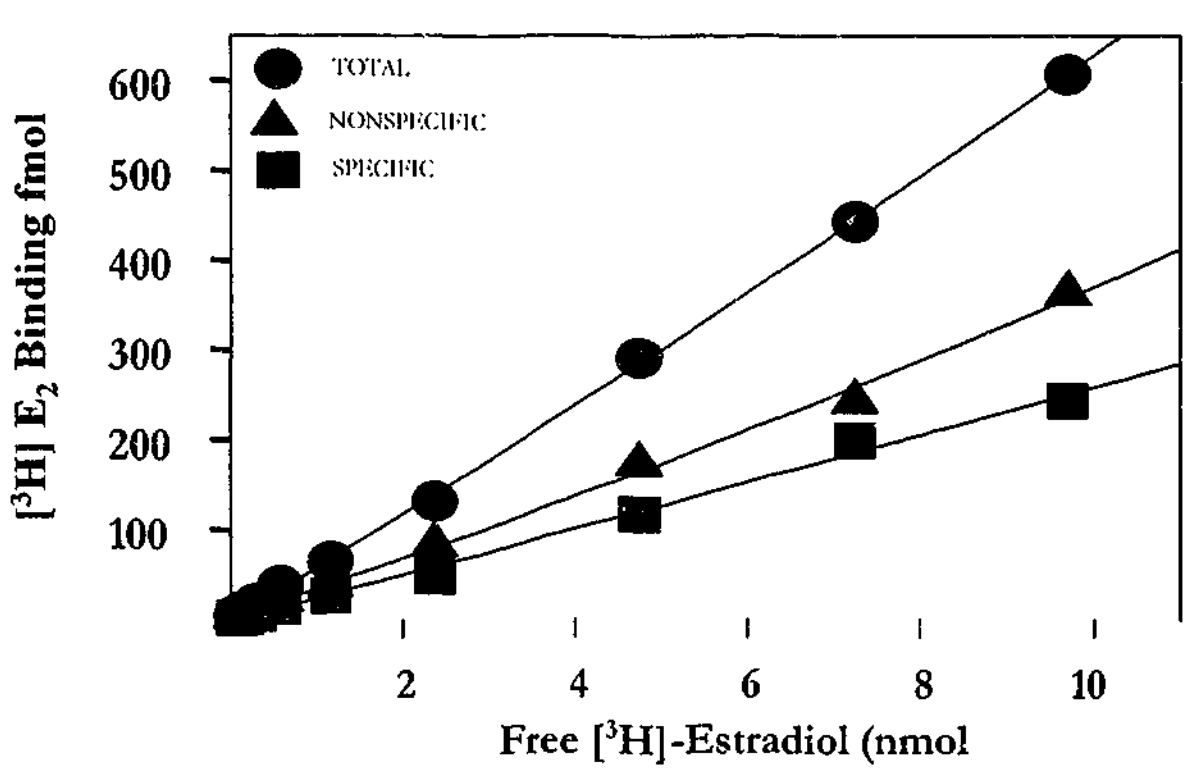


Figure 3B.3 Scatchard Analysis of Estrogen Binding in Endothelial Cells

Scatchard analysis of [^{3}H]-17β-estradiol (0.315-5 nmol/L) binding with and without 500-fold excess of nonradioactive diethylstilbestrol showing that there is no high affinity, limited capacity [^{3}H]-17β-estradiol binding consistent with estrogen receptors demonstratable in cultured vascular endothelial cells.

n = 4

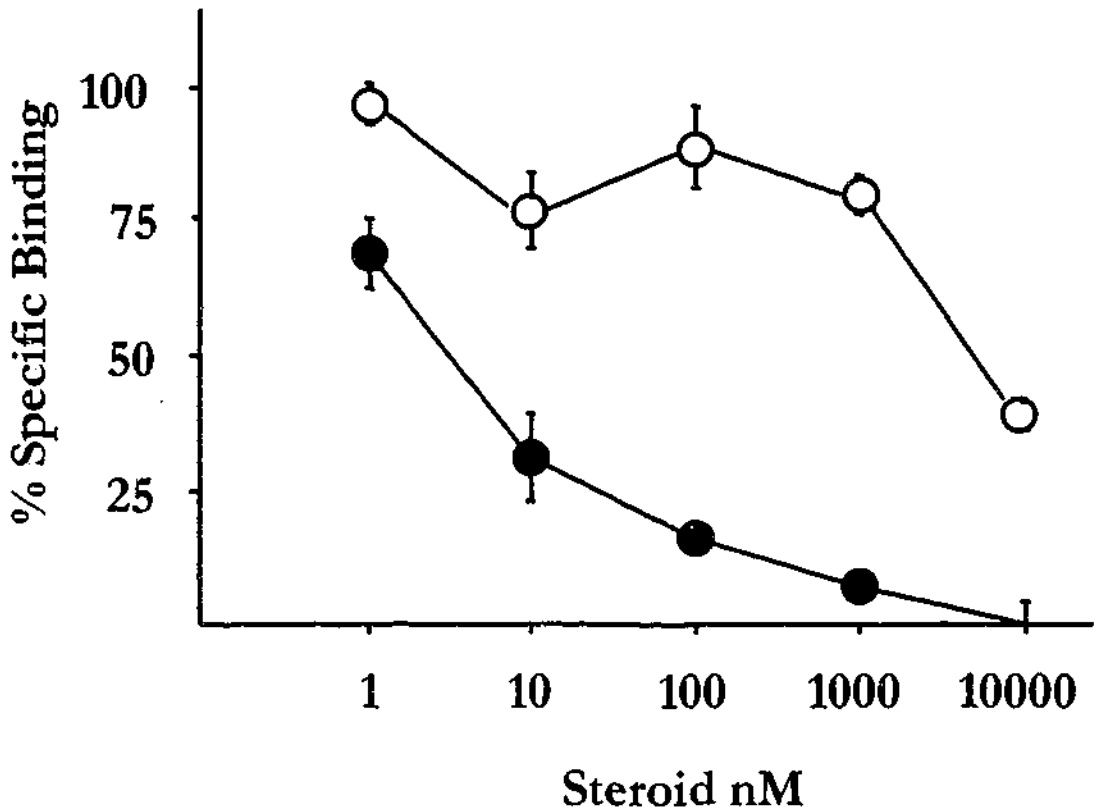
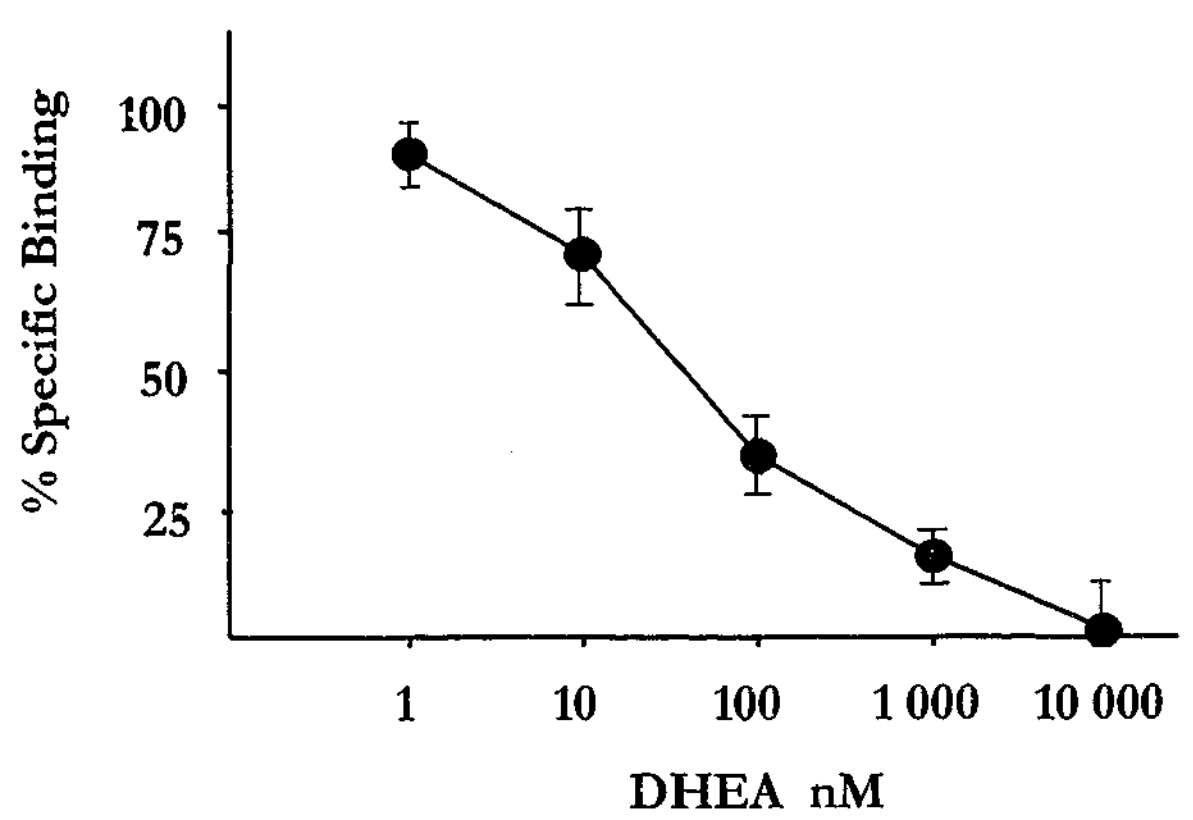
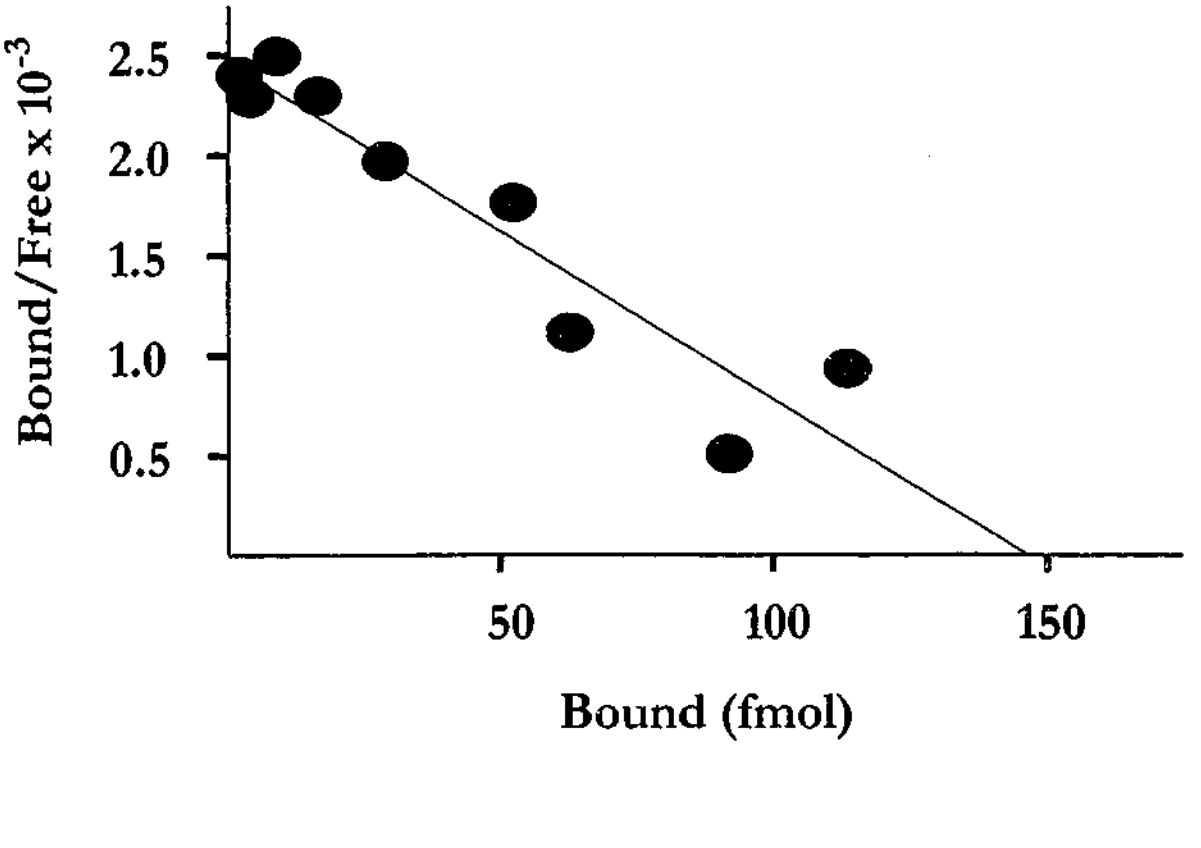


Figure 3B.4 DHEA Binding to AR in Endothelial Cells

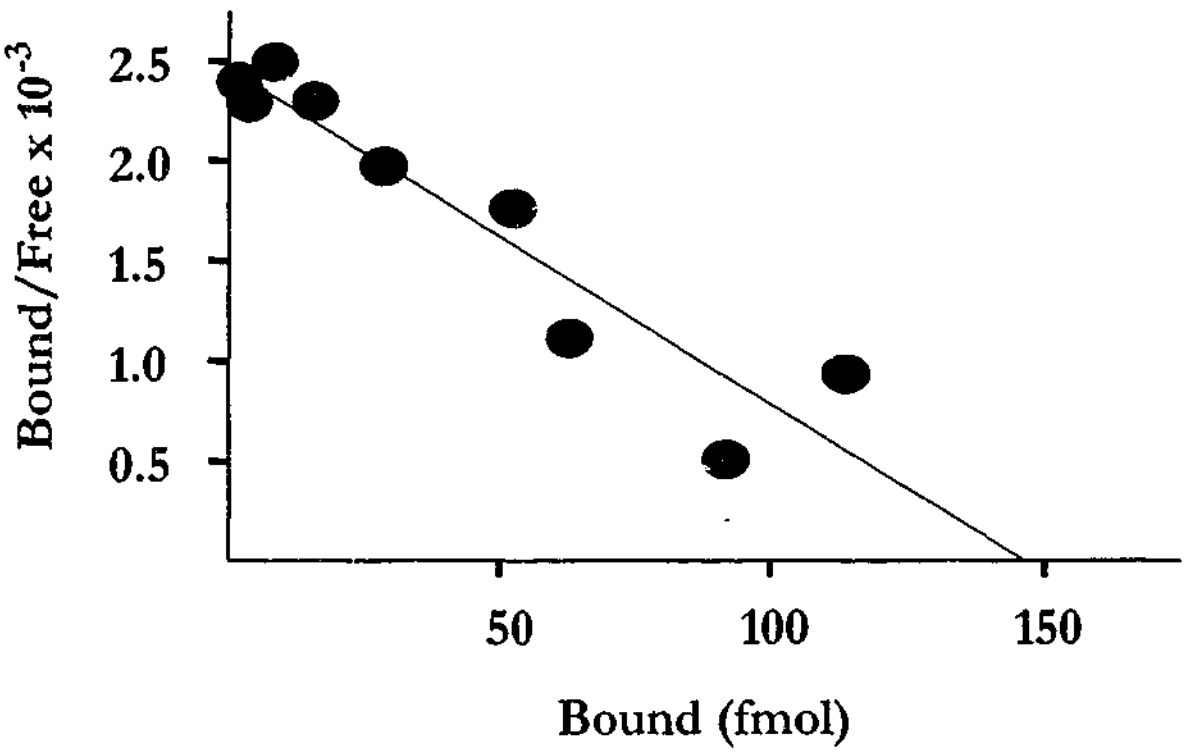
Displacement curves showing the presence of saturable binding sites for testosterone by the ability of nonradioactive testosterone to compete for binding with [3H]-R1881 (1nM) (—●—); non-specific binding (in the presence of 1 μM testosterone) varied from 60% to 75% of total. DHEA (1 – 1000 nM) (—○—) showed no displacement of 1 nM [^{3}H]R1881 binding to AR.

n = 7, with 3 replicates in each experiment


% Specific Binding
100
75
50
25
1
10
100
1 000
10 000
DHEA nM


Bound/Free x 10-3
2.5
2.0
1.5
1.0
0.5
50
100
150
Bound (fmol)

B


Bound/Free x 10^{-3}
2.5
2.0
1.5
1.0
0.5
50
100
150
Bound (fmol)

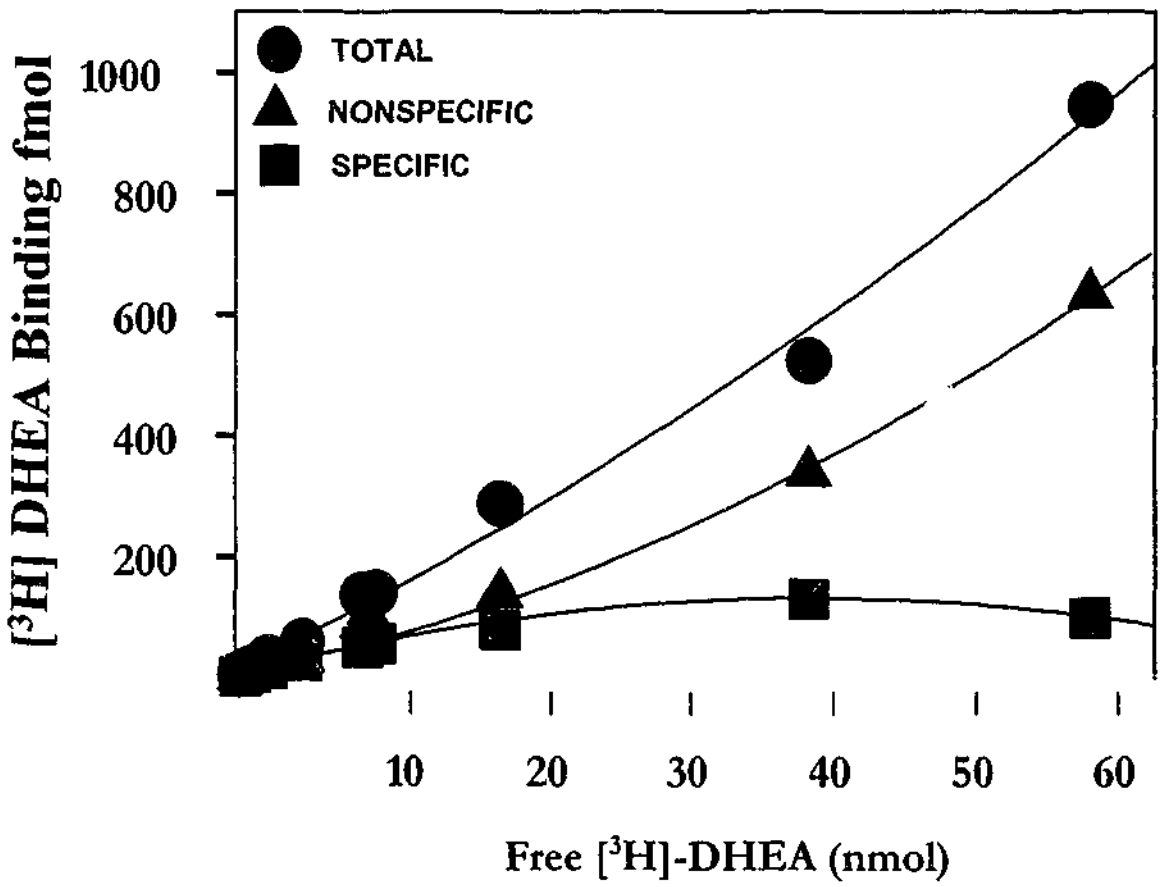

[^{3}H] DHEA Binding fmol
1000
800
600
400
200
TOTAL
NONSPECIFIC
SPECIFIC
10
20
30
40
50
60
Free [^{3}H]-DHEA (nmol)

Figure 3B.5 Specific DHEA Binding in Endothelial Cells

A. Displacement curve showing the presence of saturable binding sites for DHEA by the ability of nonradioactive DHEA to compete for binding with [3H]DHEA (10nM); non-specific binding (in the presence of 10 μM DHEA) varied from 50% to 65% of total.

B. Scatchard analysis of specific [3H]DHEA (1.4-49nM) binding with and without 500-fold excess of nonradioactive DHEA..

n = 6

3B.3 DISCUSSION

The present study has provided direct evidence in support of a specific functional role for the hormone DHEA in regulating endothelial cell behavior through direct interactions with putative DHEA receptors. Consistent with the observation that DHEA-induced increases in endothelial cell proliferation are mediated by mechanisms independent of AR and ER (Chapter 3A), we have demonstrated that specific binding of DHEA to intact endothelial cells in a manner consistent with steroid hormone receptor-like interactions.

The presence of AR in the endothelial cells was determined by Western blot. As expected, saturation analysis showed specific binding of testosterone to AR, with a binding affinity (Kd 37°C) of 10 nM. DHEA, at concentrations of 1000 nM, was unable to compete for testosterone binding to AR. Although some competition was observed at DHEA concentrations of 10,000 nM.

The presence of ERα and, to a lesser extent, ERβ in the endothelial cells was confirmed by Western blot. However, it was interesting, and somewhat surprising that Scatchard analysis failed to demonstrate specific estradiol binding in these cells. Possible explanations for this observation include: (i) increased artifact (non-specific binding), perhaps due to degradation of the estradiol used in the assay, (ii) occupation of ER by other compounds present in the cell culture media, or (iii) that endothelial cell receptors show structural or functional modifications that significantly reduce the capacity of estrogen to bind to them.

It is unlikely that the compounds used in the study were degraded or functionally inactive, as evidenced by studies conducted in our laboratory demonstrating high affinity ER binding using the same compounds in other cell lines. Furthermore, the occupation of ER by other compounds is improbable, given the repeated incubations and washing techniques used in the study. It is possible, however, that the affinity of estrogen for ER is significantly reduced in these cells. This hypothesis is supported by the observation that estradiol only stimulated endothelial cell proliferation at concentrations greater than 10 nM (Chapter 3A). Classically, estradiol is able to induce endothelial cell proliferation at concentrations of less than 0.1 nM (Concina, 2000), over two orders of magnitude less than our findings. Importantly, the finding that ICI 182,780 abolished the stimulatory effects of estradiol on endothelial cell proliferation is further evidence that the observed effects are mediated through functioning ER, and not by receptor-independent mechanisms.

We have previously shown the presence of specific DHEA binding sites in VSMC (Chapter 2B). The study reported here also suggests the existence of a putative DHEA-receptor in endothelial cells. The affinity of this binding (Kd 37°C 19 nM), measured by saturation analysis and shown in Fig. 3B.4b, is consistent with that of DHEA binding in VSMC (Kd 37°C 14nM). The observation that endothelial cells express putative DHEA-binding complexes and respond to DHEA supports the hypothesis that DHEA directly influences the behavior of vascular endothelial cells through direct interactions with cell receptors rather than through conversion to androgens or estrogens.

Part 3C

Sub-Cellular, Regulatory Mechanisms of DHEA Action In Endothelial Cells: A Possible Role for MAP-Kinase and eNOS

3C.1 METHODS

3C.1.1 Cell culture for protein kinase assays

BAEC were grown to near confluence, serum deprived for 24 hrs, and treated with DHEA (0.1 – 100 nM) for 4 hrs and then with FBS (2.5%) for 15 min. Cell protein extraction was carried out as described in Chapter 2C and protein content was measured by the Bradford method.

3C.1.2 ERK-1/2 protein kinase Western blot assays

ERK-1/2 protein kinase activity was determined by Western blot analysis. Phospo-p44/42 MAP-kinase proteins were visualized using a monoclonal phospo-p44/42 MAP kinase (Thr202/Tyr204) antibody (New England Biolabs, Beverly, MA), as described previously (Chapter 2.1.2). For relative quantification, bands were scanned in a PowerLook Scanner.

3C.1.3 Experimental Protocol for Endothelial Nitric Oxide Synthase (eNOS) Expression

Human umbelical vein endothelial cells (HUVEC)

The human endothelial cell line, EA.hy 926, was kindly donated by Dr. CJS Edgell (Department of Pathology, University of North Carolina, Chapel Hill, USA). This is a hybrid cell line resulting from the fusion of primary human umbilical vein endothelial cells with cells derived from the continuous human line A 549, originally obtained from a lung adenocarcinoma (Lieber 1976), as previously described (Edgell, 1983). Cells were cultured and maintained in DMEM containing 10% FBS and HAT.

EA.hy 926 cells were grown to near confluence, serum deprived for 24 hrs, and treated with either DHEA (100 nM) for 8, 16 or 24 hrs or DHEA (0.1 - 100 nM) for 24 hrs. Cell protein extraction was carried out as described in Chapter 2B and protein content was measured by the Bradford method. eNOS expression was analyzed by Western blot as previously described using a monoclonal eNOS antibody (Santa Cruz Biotechnology, Inc., CA, USA). For relative quantification, bands were scanned in a PowerLook Scanner.

3C.1.4 Statistical analysis

All data are presented as mean ± SEM. Statistical analysis between two observations was by Student's t-test and in multiple comparisons by analysis of variance. The null hypothesis was rejected at $p>0.05$.

3C.2 RESULTS

3C.2.1 Effects of DHEA on ERK-1/2 Activity

DHEA (1 – 100 nM) significantly stimulated FBS induced increases in ERK1/2 kinase activity with a maximum stimulation of 152±17% (10nM) compared to control (Fig 3C.1).

3C.2.2 Effects of DHEA on eNOS Expression

DHEA (1 – 100 nM) significantly increased eNOS expression in a dose dependent manner with a maximum time dependent stimulation of 174±15% compared to control (Fig 3C.2a). A time dependent increase in eNOS expression was also observed with a maximum response of 177±9% following 16 hr treatment (Fig 3C.2b).

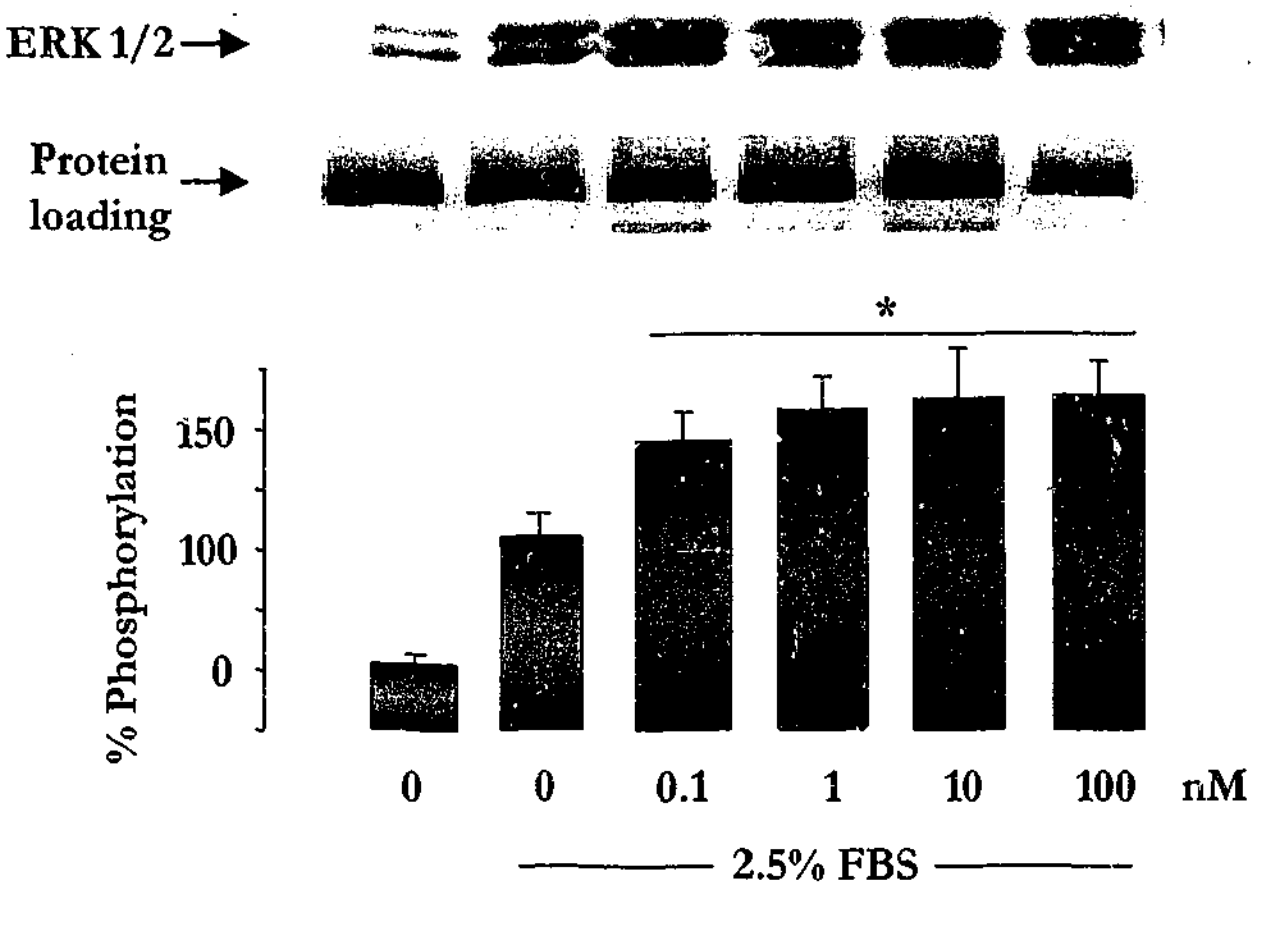


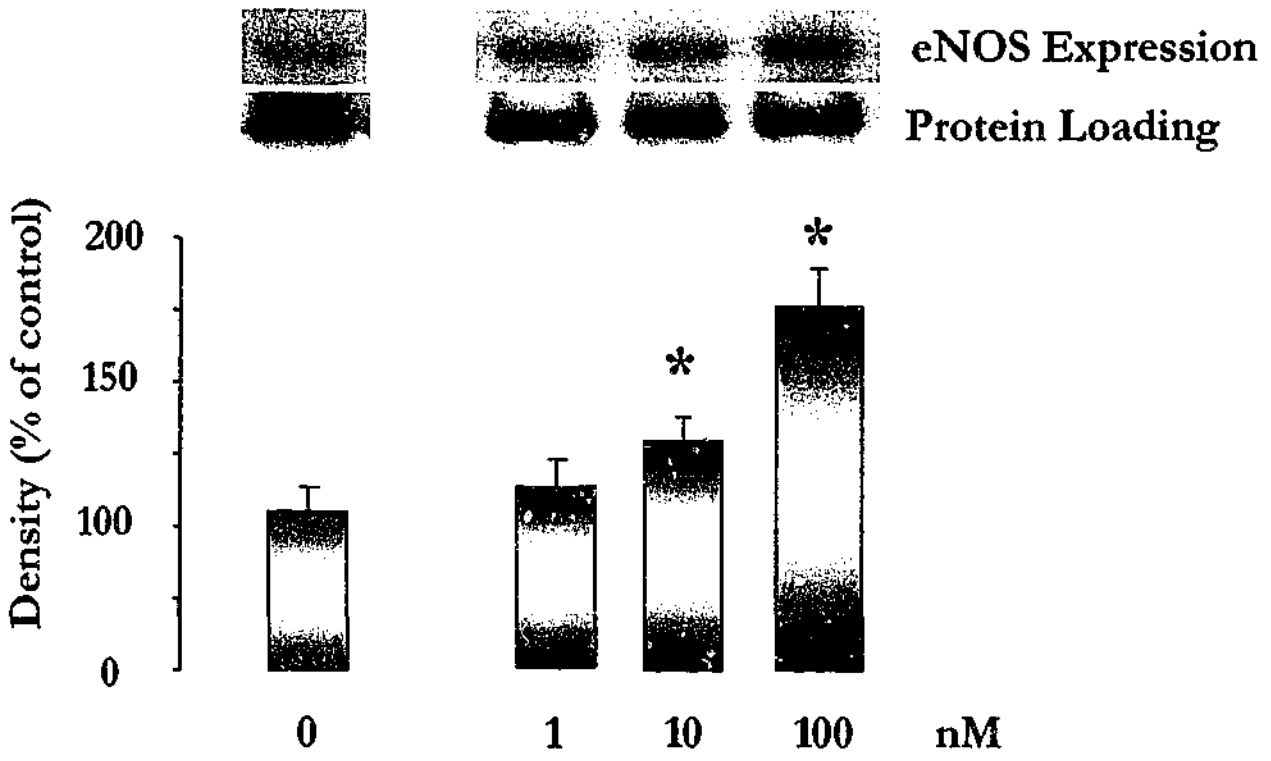
Figure 3C.1 The effects of DHEA on ERK1/2 kinase phosphorylation

DHEA (1 – 100 nM) significantly stimulates FBS induced increases in ERK1/2 kinase activity with a maximum stimulation of 152±17% (10nM) compared to control

Data are presented as mean percentage of FBS stimulation of ERK1/2 phosphorylation ± SEM.

* denotes a significant difference from FBS induced ERK1/2 phosphorylation in cultured endothelial cells.

n = 4


eNOS Expression
Protein Loading
Density (% of control)
200
150
100
0
*
*
0
1
10
100
nM

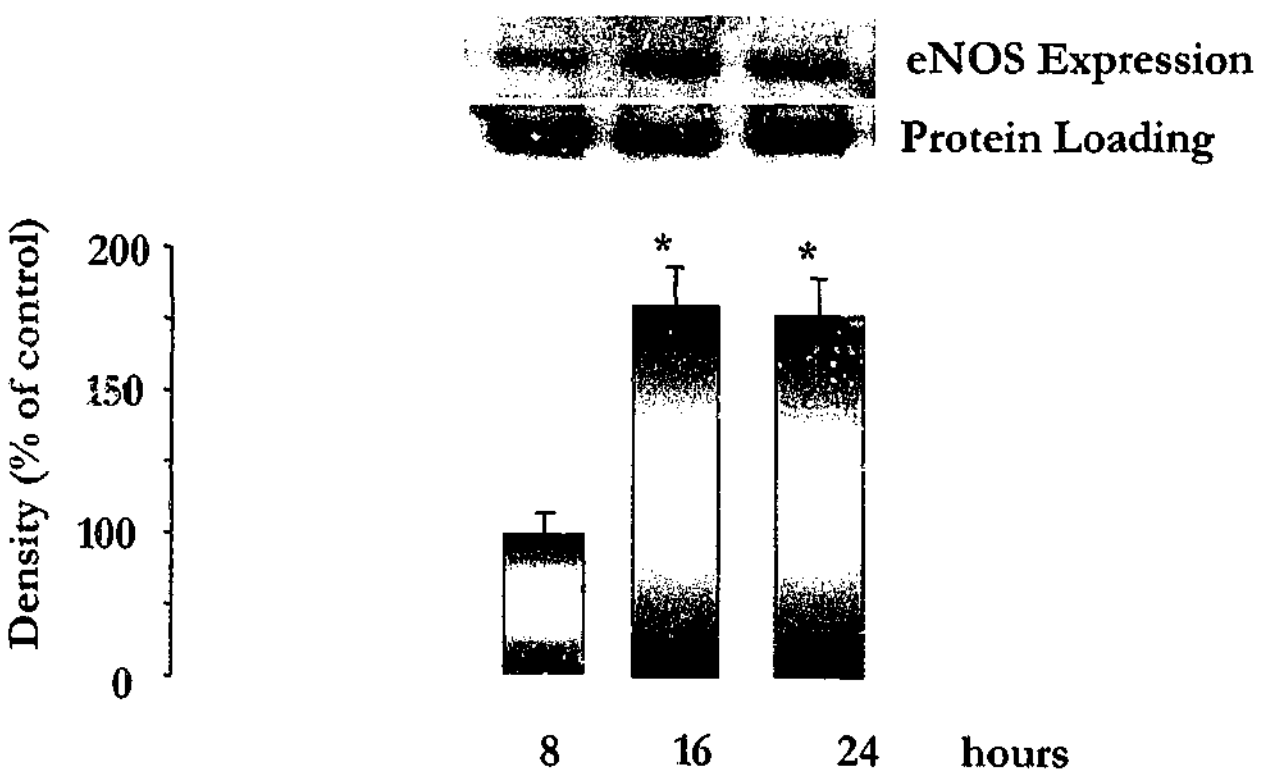

eNOS Expression
Protein Loading
Density (% of control)
200
150
100
0
*
*
8
16
24
hours

Figure 3C.2 The effects of DHEA on eNOS expression

A. DHEA (1 – 100nM) significantly increases eNOS expression in a dose dependent manner with a maximum time dependent stimulation of 174±15% compared to control.

B. DHEA (100nM, 8 – 24hr) significantly increases eNOS expression in a time dependent manner with a maximum stimulation of 177±9%(16hr) compared to control.

Data are presented as mean percentage of eNOS expression ± SEM.

* denotes a significant difference from control.

n = 4

3C.3 DISCUSSION

MAP kinases are activated in response to a variety of cell growth signals. They transduce extracellular signals from multiple membrane receptors to intracellular targets, including transcription factors, cytoskeletal proteins and other enzymes, thereby mediating a variety of cellular functions. In this study, we describe the ability of DHEA to increase the activity of the MAP kinases ERK1 and ERK2, in endothelial cell cultures. ERK1 and ERK2 are proteins of 43 and 41 kDa respectively that are approximately 85% homologous overall, with greater homology in the regions involved in binding substrates (Boulton, 1990; Boulton, 1991). They are expressed ubiquitously, although their relative abundance in different tissues is variable (Lewis, 1998).

Estrogenic activation of MAP kinase signaling pathways, leading to angiogenesis, is well documented in endothelial cells (Chen, 1999; Razandi, 2000; Russell, 2000). However, the effects of androgens on endothelial cell proliferation and sub-cellular signaling pathways are not known. To the best of our knowledge, this is the first study to provide direct evidence for DHEA-mediated stimulation of endothelial cell angiogenesis (Section 4A). In addition, our findings implicate MAP kinase signaling pathways as a potential mechanism for the proliferative effects of DHEA in endothelial cells, as evidenced by DHEA-induced ERK-1 and ERK-2 phosphorylation.

Produced primarily by endothelial cells, NO is known to play a key role in regulating vascular function. NO potentiates vasodilation of blood vessels, inhibits VSMC proliferation and promotes endothelial cell proliferation and migration. In addition, a

recent study by Bao et al. (2001) has shown that NO production and endothelial cell proliferation are mediated via ERK1/2 kinase signaling pathways (Bao, 2001). Estrogen is known to stimulate NO production in endothelial cells, via activation of eNOS mediated by its actions on ER (White, 1997; Chen, 1999). Furthermore, studies in isolated plasma membranes from pulmonary artery endothelial cells have demonstrated that stimulation of eNOS by estrogen is dependent on the activation of the MAP kinase pathway (Chen, 1999).

An increase in NO production has been recently reported following DHEA administration to pregnant women *in vivo* (Manabe, 1999). In addition, DHEA has been shown to retard atherosclerosis formation possibly by increasing NO synthesis (Hayashi, 2000). The present study, however, is the first to investigate the effects of DHEA on vascular endothelial NO production on a cellular level. We have demonstrated that DHEA significantly increases eNOS expression in endothelial cells, in a dose and time dependent manner.

3.2 CONCLUSIONS

The series of experiments described in this chapter has investigated the effects of DHEA on the proliferation of cultured vascular endothelial cells. It has provided evidence that DHEA stimulates endothelial cell angiogenesis by mechanisms independent of either androgen or estrogen receptors. Evidence has also been presented suggesting that the effects observed may be mediated via direct interactions between DHEA and DHEA-specific binding complexes in these cells.

We have also shown that DHEA significantly stimulates ERK 1/2 kinase activity and eNOS expression in vascular endothelial cells. Taken together, these results may have significant implications for the understanding of the potential effects of DHEA on vascular wall remodeling, including reendothelialization and VSMC behavior by promoting vessel wall healing and thereby decrease atherosclerosis. The observation that DHEA stimulates eNOS expression in endothelial cells suggests that DHEA may also have a vasoprotective role in the arterial system.

DHEA & The Vasculature

~Clinical Approaches~

TABLE OF CONTENTS

Chapter 4

The Effects of DHEA on Arterial Stiffness and Blood Pressure in Healthy Postmenopausal Women

4.1 INTRODUCTION

It is well established that at all ages women have a lower incidence of CAD than men. However, following menopause, CAD becomes the single greatest cause of mortality in women. Female sex hormones, principally estrogens, have been suggested to reduce the incidence and progression of CAD in postmenopausal women (Stampfer , 1991; Nabulsi, 1993), with direct effects on the lipid profile as well as on arterial mechanical properties (Rajkumar, 1997) (Reviewed in Chapter 1).

Systemic arterial compliance (SAC) is emerging as a potential index of arterial function and wellbeing, with several studies showing that hypertension, myocardial infarction and mortality are associated with increased arterial stiffening, as a result of ageing and the development of CHD (Darne, 1989). The resultant stiffening of the vasculature is associated with a number of potentially detrimental hemodynamic consequences brought about by the widening of pulse pressure, through an elevation in systolic pressure and a reduction in diastolic pressure (Chapter 1.5.1.2). Furthermore, there is evidence to suggest that pulse pressure, a surrogate measure of arterial stiffness, is an independent predictor of cardiac mortality (Darne, 1989; Fang, 1995).

Studies have demonstrated that arterial compliance is consistently higher in women than in age-matched men (Laogun, 1982). However, during the menopausal transition, arterial stiffness is greatly increased in women and gender differences are no longer prominent (Laogun, 1982; Karpanou, 1996).

As discussed in Chapter 1, an increasing body of data has supported a potentially beneficial role of estrogens on the maintenance of cardiovascular health in postmenopausal women (Stampfer, 1991; Nabulsi, 1993; Sullivan, 1996). These include pronounced effects on pulse pressure and large artery stiffness (Giraud, 1996; Liang, 1997; Rajkumar, 1997; Waddell, 1999) (Chapter 1.5.1.3). Androgens have also been implicated in the maintenance of large arterial function, although data are conflicting. A recent study, for example, has reported that androgen deprivation in males is associated with decreased central arterial compliance and reduced central systolic blood pressure (Dockery, 2000). The authors of this study concluded that while androgen withdrawal is associated with a reduction in central arterial compliance it does not affect peripheral arterial elasticity. Other studies, however, have demonstrated potentially negative effects of testosterone on the coronary vasculature (Adams, 1995; Sarrel, 1998).

Although both the occurrence and the clinical manifestation of coronary atherosclerosis have been correlated with plasma levels of DHEA, the physiological effects of the hormone remain controversial. Further, the underlying mechanisms by which DHEA might potentially act on specific cardiovascular risk factors such as arterial elasticity and pulse pressure are not known. In addition, despite the existence of *in vitro* data suggesting a potential atheroprotective role for DHEA (Chapters 2 and 3), it is not known whether these effects are translated into the clinical setting.

Aims of Study

To address these issues, the current study employed a double-blind, placebo controlled design to investigate the effects of oral DHEA supplementation on large artery stiffness and pulse pressure in healthy postmenopausal women.

4.2 METHODS

4.2.1 Subjects

Thirty six healthy, postmenopausal, normotensive, nondiabetic, non-smoking women, aged 42 to 70 years, and not taking any form of hormonal therapy or other medication likely to affect vascular function (although several subjects on long-term stable doses of antihypertensive drugs were included), were recruited by advertisement from the general community. All subjects gave written informed consent for their participation in the study, which was approved by the Alfred Hospital Ethics Review Committee. Prior to participation in the study, subjects underwent a screening process to ensure their suitability.

4.2.2 Study Design

A longitudinal double-blind, placebo controlled study design was employed to investigate the effects of three months of DHEA administration on large artery mechanical properties in health postmenopausal women. The subjects were randomized into two groups of eighteen one of which received active treatment (DHEA, 100 mg daily) and the other placebo. Randomization was carried out by an independent member of the institute to ensure that the investigators, operators and subjects were blinded throughout the duration of the study. Subjects were requested to refrain from the consumption of food and drink for a minimum of 8 hours prior to the study. They attended the Menopause Clinic at the Baker Medical Research Institute on two occasions for testing: the first time for baseline measurements and the second following three months of either DHEA or placebo treatment.

On each occasion, the following procedures were performed: height and weight measurements, resting blood pressure, heart rate and arterial stiffness. In addition, a venous blood sample was obtained for the measurement of serum hormone levels (DHEA, estradiol and testosterone) and lipids (triglycerides, HDL, LDL and total cholesterol).

4.2.3 Anthropometric Measurements

Each subject's height and weight were measured, without shoes, on the day of testing and body mass index (BMI) was calculated.

4.2.4 Biochemical Analysis

Fasting venous blood samples were drawn on the day of testing for analysis of lipids and hormone levels.

4.2.5 Lipids

Total, low-densisty lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and total triglycerides were determined enzymatically (Boehringer Mannheim/Hitachi 917 Analyser) by the Alfred Hospital Pathology Service (Melbourne, Australia).

4.2.6 Hormones

Venous blood samples were obtained from subjects for the quantitative determination of the steroid hormones DHEA, estradiol, estrone and serum hormone binding globulin

(SHBG) and testosterone. Blood samples were batched for all assays. Plasma Hormone levels were measured by Mayne Health, Dorevitch Pathology (Heidleberg, Melbourne Australia) using commercial radioimmunoassay kits to measure plasma DHEA (ICN Pharmaceuticals, CA, USA), estradiol (Orion Diagnostica, Finland), estrone (Diagnostics Biochem Canada Inc, Canada), SHBG (Bayer Delfia) and testosterone (Biosource Europe, SA) levels. Free androgen index (FAI) was then determined using the following equation:

FAI = Total testosterone/SHBG

4.2.7 Measures of Arterial Stiffness

4.2.7.1 Systemic Arterial Compliance (SAC)

Compliance of the total systemic arterial system was determined by calculations based on the two-element Windkessel model of blood circulation and the area method as described by Liu et al (Liu, 1986), which requires the simultaneous measurement of ascending aortic blood flow and associated carotid blood pressure.

4.2.7.1.1 Flow

Volumetric ascending aortic flow velocity was assessed by a hand held Doppler velocimeter (multi-doplex MDI, Huntleigh Technology, Cardiff, UK) placed in the suprasternal notch (Figure 4.1 - A). A Doppler shift is relayed by the velocimeter as a result of the backscatter from moving erythrocytes within the measured volume and an analogue signal proportional to the frequency determined by the number of detected zero crossings per unit time is obtained. This signal is then related, via the Doppler equation, to flow in the ascending aorta.

The derived flow values obtained by this method are generally lower than those obtained invasively and as such are expressed as arbitrary compliance units (a.c.u) (dimensionally equivalent to ml/mmHg). The position and angle of the flow velocimeter on the suprasternal notch was determined by obtaining wave forms with the highest systolic peak.

4.2.7.1.2 Pressure

Aortic root driving pressure was determined by applanation tonometry of the proximal right carotid artery using a calibrated non-invasive Millar Mikro-Tip pressure transducer (model SPT-301, Miller Instruments, Houston, Texas). Carotid pressure wave forms were obtained by placing the pressure transducer on the carotid artery as the patient lay in the supine position with the head tilted back (Figure 4.1 - B). The pressure transducer was placed on the carotid artery at an angle enabling the visualization of optimal carotid pressure wave forms free of distortions and with the highest possible systolic peak.

The pressures obtained by this method are calibrated against brachial mean arterial blood pressure measurements made simultaneously with a Dynamap vital signs monitor (1846SX, Critikon, Florida, USA). The calibration method assumes that both mean arterial blood pressure (MAP) and end-diastolic blood pressure remain constant throughout the vasculature, and therefore, central systolic blood pressure and central pulse pressure could be calculated. Following the manual input of the mean of three consequtive brachial blood pressure measurements, analysis of central systolic blood

pressure and central pulse pressure was achieved using purpose written software (Pascal Turbo, Borland International, Inc.).

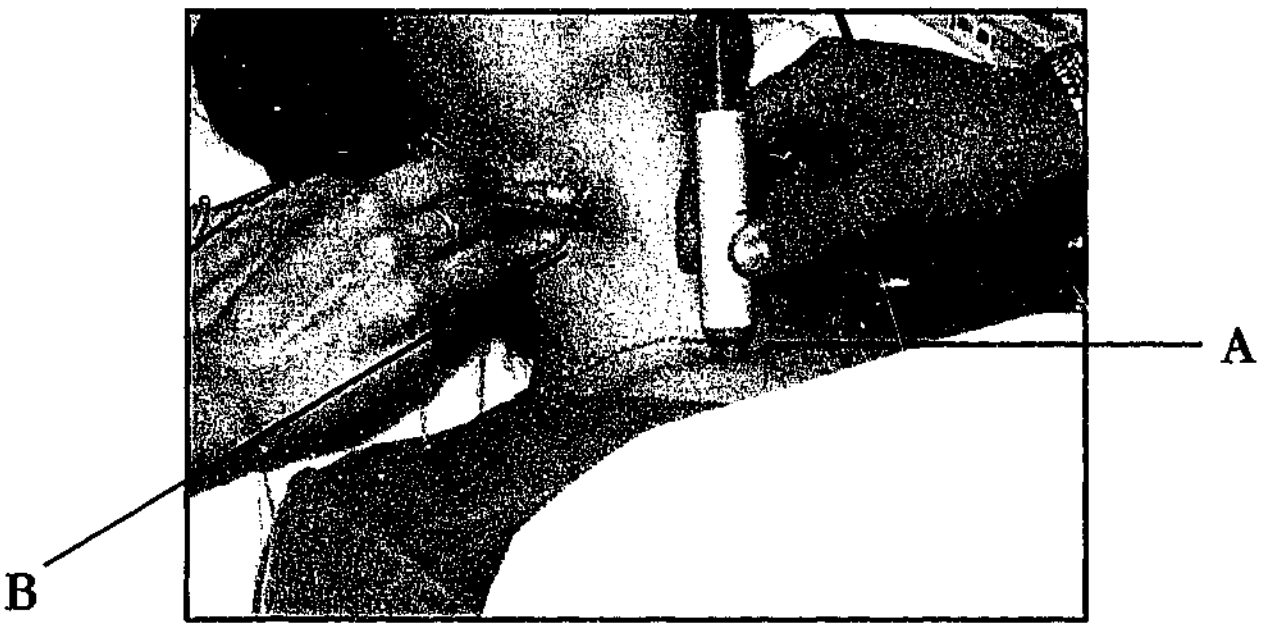


Figure 4.1 Positioning of Flow and Pressure Probes in the Measurement of SAC

A Doppler velocimeter placed on the suprasternal notch to measure ascending aortic blood flow.

B Pressure transducer placed on the carotid artery to measure carotid blood pressure

4.2.7.2 Analysis

SAC was calculated using the following equations:

$$SAC = Ad / [R (Ps - Pd)] \qquad R = MAP/Mean\ systolic\ flow$$

Ad = area under the diastolic portion of the blood pressure wave form (from end systole to end diastole) (Figure 4.2 - A)

R = total peripheral resistance (TPR)

Ps = end systolic blood pressure

Pd = end diastolic blood pressure

MAP = mean arterial pressure = area under the pressure wave form divided by the time interval ($t_2 - t_1$) (Figure 4.2 - B)

Mean systolic flow was obtained from the Doppler flow velocimeter signal

Flow and pressure signals were digitized at 200 Hz using a Data translation DT 2801 analogue-to-digital board. Analysis of SAC was achieved using purpose written software (Pascal Turbo, Borland International, Inc.) following the selection of 10 representative flow and pressure wave forms. The operator was required to select consistent representative traces with stable baselines, and define the end systolic and end diastolic points prior to computer analysis.

4.2.8 Statistical analysis

All data are presented as mean ± SEM. Statistical analysis between two observations was by Student's t-test and in multiple comparisons by analysis of variance. The null hypothesis was rejected at $p>0.05$.

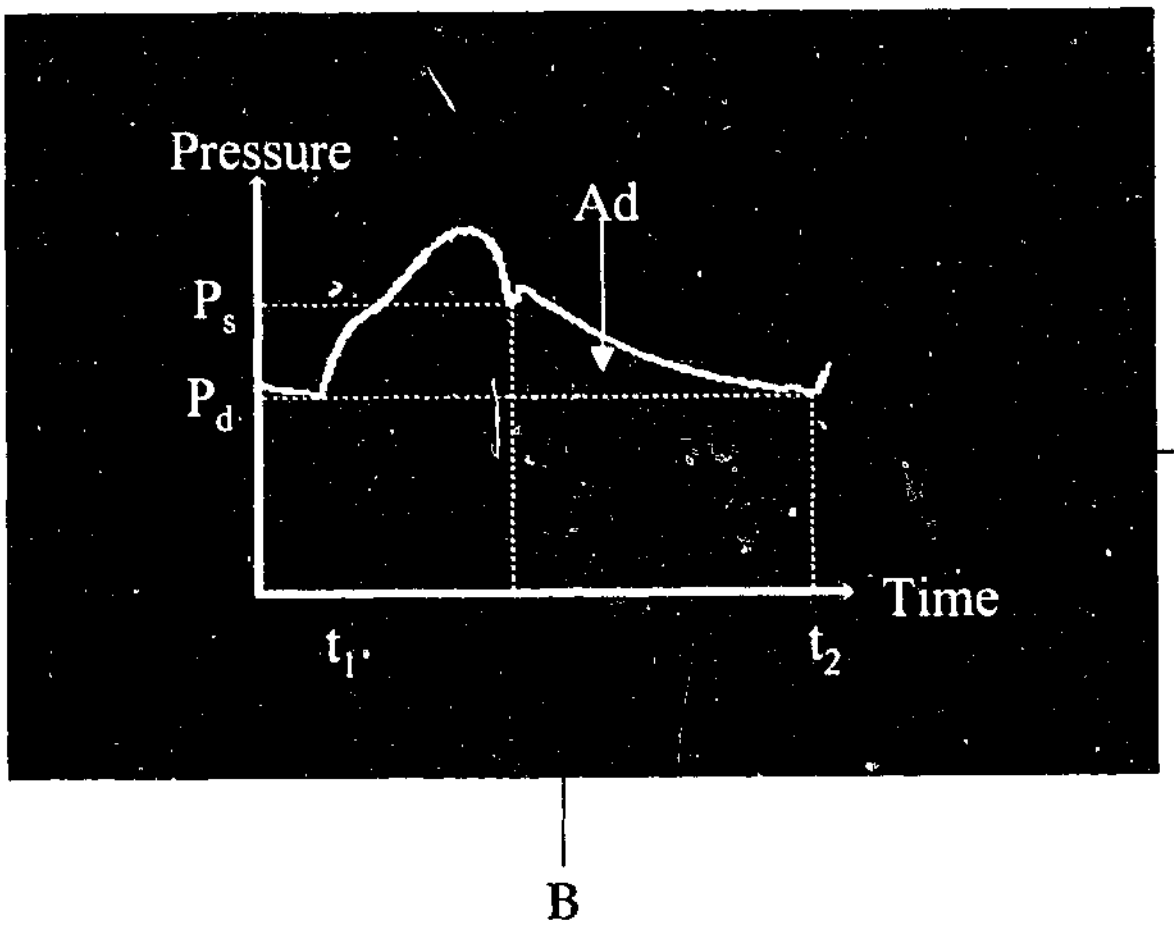


Figure 4.2 SAC Analysis

A Ad – the area under the diastolic portion of the blood pressure wave form (from end systole to end diastole).

B MAP – mean arterial pressure: The area under the arterial pressure wave form divided by the cardiac cycle duration ($t_2 - t_1$).

4.3 RESULTS

4.3.1 Subject characteristics

As Table 4.1 illustrates, subjects in both treatment groups were well matched for the baseline characteristics that are known to affect mechanical arterial properties. While there were no significant differences in any of the variables measured.

4.3.2 Effects of DHEA on systemic arterial compliance

There were no significant differences in baseline arterial compliance values between the two treatment groups (0.12 ± 0.01 and 0.12 ± 0.01 a.c.u for DHEA and placebo treated groups respectively). However, in the DHEA treated group, arterial compliance increased significantly to 0.18 ± 0.02 a.c.u, $p<0.05$ following 12 weeks of DHEA administration. There were no changes in SAC following 12 weeks of placebo. Fig 4.4 shows the effects of 12-week supplementation of DHEA on SAC compared with those of placebo.

4.3.3 Effects of DHEA on plasma lipids

As Table 4.2 illustrates, there was a significant decrease in total cholesterol following 12 weeks of DHEA supplementation (6.2 ± 0.2 to 5.5 ± 0.2, $p<0.01$). There were no differences observed in total plasma, HDL, or LDL cholesterol levels in the placebo treated group (6.0 ± 0.2 to 5.8 ± 0.2, $p=0.14$). The decrease observed in total cholesterol levels in the DHEA treatment group subjects was coupled with no significant change in LDL (3.5 ± 0.3 Vs 3.3 ± 0.2) or HDL levels (1.9 ± 0.1 Vs 1.6 ± 0.1) ($p=0.14$ and $p=0.1$

respectively). There were no changes in LDL or HDL levels following placebo treatment. HDL/LDL ratios were not significantly altered in either treatment group (0.58 ± 0.1 to 0.54 ± 0.1, p=0.08, and 0.48 ± 0.02 to 0.5 ± 0.04, p=0.3, for DHEA and placebo respectively).

4.3.4 Effects of DHEA on hormone levels

As expected, plasma DHEA levels significantly increased following DHEA administration (5.4±0.8 to 11.5±1.7ng/mL, $p<0.05$). In addition, while plasma estrone levels significantly increased (10.7±1.6 to 28.4±4.6pmol/L, $p<0.05$) there were no changes in plasma estradiol levels (58.7±19.9 to 59.6±11pmol/L) following DHEA administration. Testosterone levels significantly increased (0.6±0.05 to 2.4±0.8nmol/L, $p<0.05$) whereas serum hormone binding globulin (SHBG) levels did not significantly change following DHEA administration (37.9±2.9 to 30.3±3.3nmol/L, p=0.09). Free androgen index (FAI), a rough index of free testosterone levels, significantly increased following twelve weeks of DHEA treatment (0.02±0.0003 to 0.07±0.01, $p<0.05$). There were no changes in hormonal levels in the placebo treated group as detailed in Table 4.2.

4.3.5 Effects of DHEA on arterial pressure

Table 4.2 illustrates that there were no significant changes were observed in systolic, diastolic, mean arterial pressure (MAP) or pulse pressure values at baseline compared to 12-week DHEA administration (91.3±2.7 and 90.5±2.9 compared to 87.3±2.3 and 86.6±1.8 mmHg for DHEA and placebo treatments respectively) (p=0.117 and 0.113 for DHEA and placebo treatments repectively) as assessed by brachial artery blood pressure

measurements. Similarly, no changes were observed in systolic, diastolic and pulse pressure with placebo treatment (refer to Table 4.2). However, there was a significant reduction in MAP in the placebo treatment group (90.2±1.7 and 86.4±1.8 mmHg, $p<0.03$).

Significant differences were observed in measures of central (carotid) arterial pressures in the DHEA treatment group. Mean values for central systolic blood pressure were significantly decreased (124.5±3.6 to 112.8±3.7, $p=0.03$) and central pulse pressure was significantly reduced (55.8±3.4 to 43.7±3.6, $p=0.02$) in the DHEA treatment group. Diastolic blood pressure was unaltered following DHEA administration. There were no changes observed in central systolic, diastolic or pulse pressures following placebo treatment.

Variable	Control (Placebo) Group	DHEA Treatment Group	P
Age (years)	57.4 ± 1.9	59.2 ± 1.3	0.43
Height (m)	163 ± 2.5	163 ± 1.9	0.86
Weight (kg)	66.7 ± 3	71.5 ± 2.9	0.1
BMI ($kg \cdot m^{-2}$)	25.6 ± 0.9	27.5 ± 1.2	0.33
Total Chol (mmol/L)	6.0 ± 0.2	6.2 ± 0.2	0.39
LDL Chol (mmol/L)	3.7 ± 0.1	3.5 ± 0.3	0.41
HDL Chol (mmol/L)	1.7 ± 0.1	1.9 ± 0.1	0.38
Total Trigs (mmol/L)	1.3 ± 0.2	1.4 ± 0.2	0.26

Table 4.1 Baseline Group Characteristics

Subjects in both treatment groups were well matched for the baseline characteristics that are known to affect mechanical arterial properties. There were no significant differences in any of the variables measured.

P values represent comparisons between the two treatment groups

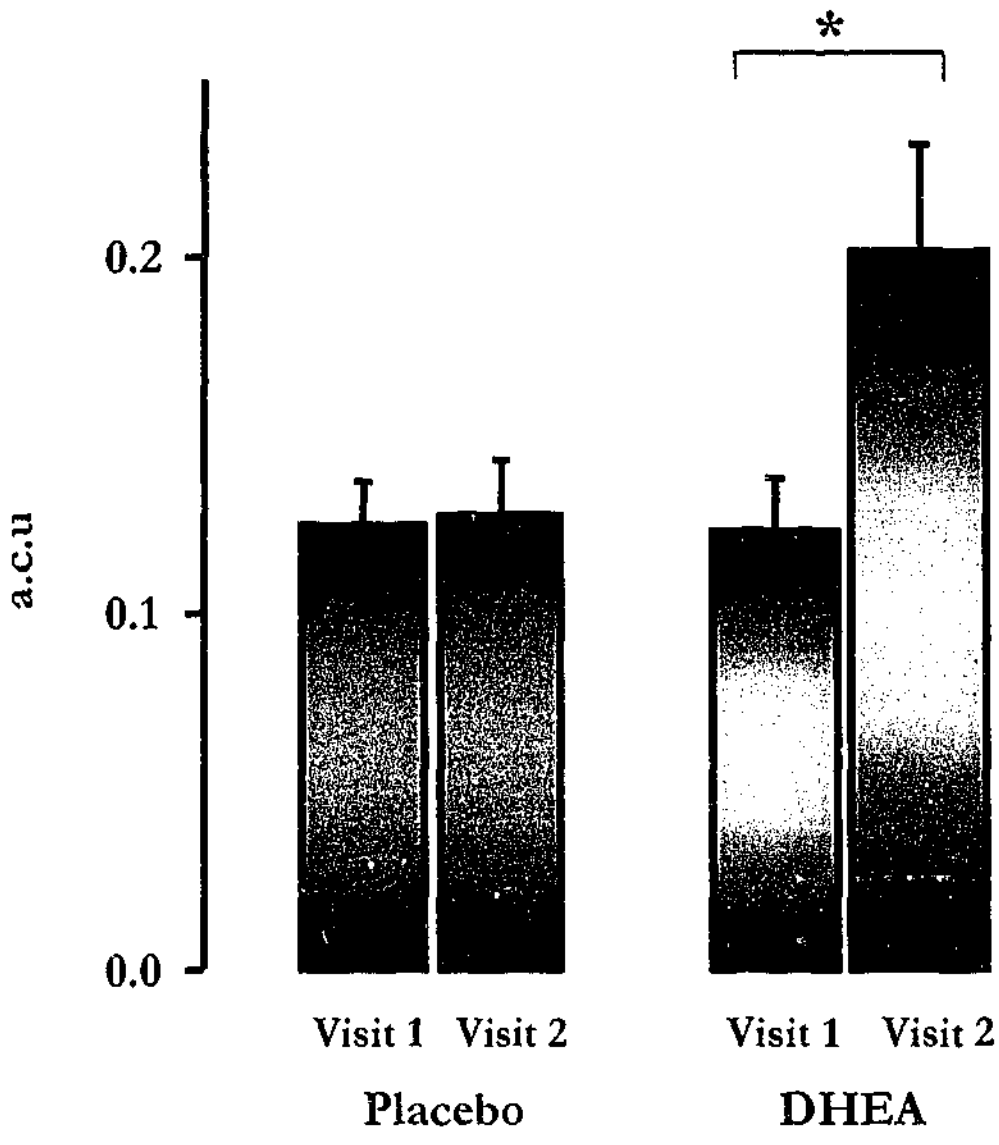


Figure 4.3 Effects of DHEA on Systemic Arterial Compliance in Healthy Postmenopausal Women

SAC was significantly increased following DHEA administration compared with baseline values (0.12 ± 0.01 compared to 0.18 ± 0.02a.c.u , $p<0.05$). There were no differences observed in SAC between measurement time points in the placebo treated group (0.12 ± 0.01 compared to 0.12 ± 0.01).

Data are presented as mean arbitrary compliance units (a.c.u) ± SEM.

* denotes a significant difference between measurement time points.

Variable	Control (Placebo) Group		DHEA Treatment Group	
	Baseline	*Intervention*	*Baseline*	*Intervention*
Lipids (mmol/L)				
Total Chol	6.0 ± 0.2	5.8 ± 0.2	6.2 ± 0.2	5.5 ± 0.2*
LDL Chol	3.7 ± 0.1	3.5 ± 0.2	3.5 ± 0.3	3.3 ± 0.2
HDL Chol	1.7 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.6 ± 0.1
HLD/LDL ratio	0.48 ± 0.02	0.5 ± 0.04	0.58 ± 0.1	0.54 ± 0.1
Total Trigs	1.3 ± 0.2	1.4 ± 0.3	1.4 ± 0.2	1.4 ± 0.2
Blood Pressure (mmHg)				
Systolic BP	117.9 ± 2.3	116.5 ± 2.8	121.1 ± 3.4	117.9 ± 3.6
Diastolic BP	69.9 ± 2.3	68.1 ± 1.6	68.6 ± 2.0	69.1 ± 2.1
MAP	90.3 ± 1.8	86.4 ± 1.8	91.3 ± 2.7	90.5 ± 2.9
Pulse Pressure	48 ± 2.9	48.4 ± 2.9	52.5 ± 2.5	48.9 ± 3.6
Central Systolic BP	117.8 ± 2.9	112 ± 3.2	124.5 ± 3.6	112.8 ± 3.7*
Central Pulse Pressure	47.9 ± 3.3	44.3 ± 3.2	55.8 ± 3.4	43.7 ± 3.6*
Hormones				
DHEA (ng/mL)	4.4 ± 0.6	4.1 ± 0.6	5.4 ± 0.8	11.5 ± 1.7*
Estradiol (pmol/L)	53.1 ± 19	48.9 ± 12.5	58.7 ± 19.9	59.6 ± 11
Estrone (pmol/L)	9.7 ± 2.5	7.6 ± 1.4	10.7 ± 1.6	28.4 ± 4.6*
SHBG (nmol/L)	46.3 ± 6.6	49.4 ± 3.2	37.9 ± 2.9	30.3 ± 3.2
Testosterone (nmol/L)	0.5 ± 0.04	0.4 ± 0.05	0.6 ± 0.05	2.4 ± 0.8*
Free Androgen Index	0.01 ± 0.007	0.01 ± 0.004	0.02 ± 0.003	0.07 ± 0.01 *
Heart Rate (beats/min)	71 ± 4	69 ± 5	69 ± 4	69 ± 5

Table 4.2

Lipid, blood pressure and plasma hormone level measurements at baseline and following 3 months of either DHEA or placebo intervention.

Data are presented as mean values ± SEM.

* denotes a significant difference between measurement time points ($p<0.05$).

4.4 DISCUSSION

This is the first study to investigate the effects of DHEA administration on arterial mechanical properties in healthy postmenopausal women. The current study demonstrated that three-month oral DHEA supplementation in postmenopausal women reduces central arterial stiffness, as measured by SAC and carotid pulse pressures, compared with placebo treatment. The observed beneficial effects on arterial mechanical properties were found to be independent of mean arterial pressure and heart rate.

The structural integrity and elastic properties of the large arteries at any point in time are dependent on the plasma lipid profile, mean arterial and pulse pressures, heart rate, body composition, fitness level and other indices of coronary risk (as discussed in Chapter 1.6.1.1). At baseline, the two groups of postmenopausal women involved in the current study were matched for the above variables (Table 4.1). In addition, neither DHEA nor placebo affected mean arterial or end diastolic pressures or heart rate. We therefore conclude that the differences observed in central arterial stiffness between those women taking DHEA and those taking placebo are not attributable to these variables.

A number of studies, but not all, have suggested that DHEA administration alters plasma lipid levels. While some studies have shown beneficial effects of DHEA on the lipid profile (Nestler, 1988; Kurzman, 1990; Haffner, 1995) others have shown no effects (Gordon, 1988; Arad, 1989; Eich, 1993) or negative effects (Mattson, 1980; Mortola, 1990; Morales, 1994; Bednarek-Tupikowska, 1995). In the current study, although total plasma cholesterol was reduced in women taking DHEA, there were no significant

changes in HDL or LDL cholesterol, or in triglyceride levels. Therefore, the increases in arterial compliance observed in those women taking DHEA are unlikely to be a consequence of changes in lipid levels.

As discussed in Chapter 1, increased arterial stiffness is associated with elevated systolic and reduced diastolic blood pressures and the consequent widening of pulse pressure. There are no studies published reporting the effects of exogenous DHEA administration on blood pressure in humans, and studies of DHEA administration in animal models of hypertension have yielded conflicting results. A study by Li et al. (1996) investigating the effects of DHEA on adrenocorticotrophin-induced hypertension in rats found no effects of DHEA on blood pressure (Li, 1996). Conversely, another study in rats found that DHEA administration abolishes dexamethasone-induced hypertension (Shafagoj, 1992).

Observational studies in humans have found that elevated systolic and diastolic blood pressures are associated with increased plasma DHEAS levels (Hautanen, 1994; Schunkert, 1999). Results from the Massachusetts Women's Health Study found the both DHEA and DHEAS were positively correlated with diastolic and systolic blood pressure. The authors of this study concluded that higher levels of DHEA and DHEAS in middle-aged women may indicate increased CVD risk (Johannes, 1999).

The current study shows no effect of DHEA on brachial systolic blood pressure. In addition, there was no effect of DHEA on mean arterial or end diastolic blood pressure. However, a reduction in carotid pulse pressure, associated with a decrease in central systolic blood pressure, was clearly evident following DHEA administration as opposed

to placebo treatment. These data suggest that the actions of DHEA in the reduction of arterial stiffness are mediated, at least in part, by a reduction in central systolic and pulse pressures but independently of mean arterial and diastolic blood pressures.

Estrogens have been suggested to exert positive effects on the coronary vasculature by inhibiting atherosclerotic progression and vascular smooth muscle proliferation (see Section 1.5.2). However, clinical and epidemiological studies have reported discordant observations (as discussed in Chapter 1). It has been proposed that the conflicting findings are a result of the type of estrogens used in the various studies. Indeed it has been shown that estrone and estriol have a significantly lower affinity for estrogen receptors than estradiol (Katzenellenbogen, 1984; Bridges, 1993). Furthermore, a recent study investigating the differential effects of estrogens on VSMC proliferation demonstrated that while 17β-estradiol significantly inhibited VSMC proliferation by a receptor mediated mechanism, estrone, estrone sulfate and estriol had no effect on VSMC proliferation (Dubey, 2000).

In the current study, we propose that the reductions in arterial stiffness as well as carotid systolic and pulse pressures reflect direct DHEA-vessel wall interactions rather than DHEA conversion to estrogens. This hypothesis is supported by the analysis of plasma hormone levels which revealed significant increases in plasma DHEA and estrone levels, but not in estradiol.

As plasma testosterone levels increased significantly in this study, the possibility of the observations being an androgenic rather than a DHEA effect cannot be dismissed. On

the one hand, androgen levels have been inversely related to carotid intimal-medial thickness in postmenopausal women (Bernini, 2001) and myocardial ischemia in men (Webb, 1999). On the other, other studies have shown no such associations (Kabakci, 1999; Price, 2000). In fact, a recent review reported that androgens appear to promote atherosclerosis in women (Sarrel, 1998). Animal studies have also shown testosterone administration to accentuate atherosclerotic progression (Adams, 1995). Adding weight to potentially negative effects of testosterone are *in vitro* studies demonstrating that testosterone increases VSMC proliferation (Fujimoto, 1994) (Chapter 2A).

It is our contention that the effects observed were not the result of changes in testosterone levels for the following reasons: although discordant findings have been reported, the weight of opinion in the literature favors a negative or null effect of testosterone in cardiovascular pathophsiology. *In vitro* findings presented in this thesis (Chapter 2) that testosterone promotes VSMC proliferation coupled with evidence from animal and human studies confirming that testosterone treatment significantly accentuates systolic and diastolic blood pressures (Chen, 1991; Oudar, 1991; Reckelhoff, 1998; Reckelhoff, 1999; Soranno, 1999), results contradictory to those of the current study demonstrating a reduction in central arterial pressures following DHEA administration, suggest that the increases SAC seen in the current study are not attributable to testosterone. Furthermore, while total testosterone levels significantly increased (with a corresponding increase in the free androgen index), they remained within the "postmenopausal range" (as specified by BioSource Europe S.A, 2001), suggesting that in absolute terms the increase in plasma testosterone levels was small and final testosterone levels was low. Whilst it is therefore probable that the observations of

this study predominantly reflect a specific DHEA-mediated effect rather than conversion to androgens or estrogens, further investigation is warranted to elucidate the precise mechanisms of DHEA action on the mechanical behavior of the large central arteries.

Previous studies have put forward two hypotheses concerning the potentially beneficial effects of sex hormones, primarily estrogens, on the reduction of arterial stiffness (Rajkumar, 1997). The first hypothesis relates to short-term vasodilatory responses in which a transfer of vascular wall stress from collagen to the more distensible elastin fibres occurs leading to more compliant vessels. The second postulation involves long-term structural vascular changes via the modulation of VSMC numbers (Vargas, 1993) and relative proportions of collagen and elastin fibers (Fischer, 1977).

The results of the current study, coupled with those previously described in Chapters 2 and 3, suggest that DHEA may increase arterial compliance by mechanisms involving the reduction of central systolic and pulse pressures, and the inhibition of VSMC proliferation (Chapter 2). Another possible mechanism by which DHEA increases arterial compliance is via the modulation of endothelial cell proliferation and regulatory functions such as the production of nitric oxide (Chapter 3). In addition, while the structural vascular changes involving the production of collagen and elastin generally reflect long-term exposure, it is possible that these structural changes may have been instigated during the three-months of DHEA administration and consequently contributed to the results of the study.

4.5 CONCLUSIONS

The present study has demonstrated that DHEA supplementation in postmenopausal women has profound effects on the mechanical properties of the central arterial vasculature. We have demonstrated that DHEA significantly attenuates central arterial stiffness and reduces central systolic and pulse pressures. The findings of this study provide direct evidence in support of a potentially atheroprotective role for DHEA in the setting of human cardiovascular physiology, and may offer some insight into the mechanisms by which DHEA may contribute to the lowering of the incidence of cardiovascular disease.

Chapter 5

The Effects of DHEA on Endothelium-Dependent Vasodilation in Healthy Postmenopausal Women

5.1 INTRODUCTION

The vascular endothelium plays an integral role in the regulation and maintenance of blood vessel function by its elaboration of a diverse array of paracrine factors (Chapter 1). Under normal physiological conditions the predominant functions of the endothelium appear to be promotion of vasodilation, and inhibition of thrombosis and cellular proliferation. However, under pathological conditions the balance of endothelial function shifts to vasoconstriction, thrombosis and smooth muscle cell proliferation, all of which are involved in the pathogenesis of vascular disease and atherosclerosis (Chapter 1.3).

The vascular endothelium contributes to cardiovascular homeostasis by regulating the calibre of blood vessels in response to changing hemodynamic and hormonal environments (Chapter 1.3.1.3). This is achieved through the production and release of vasoactive factors, which in turn alter vasomotor tone and maintain blood flow (Rubanyi, 1993). Flow-induced endothelium-dependent vasodilation of large conduit vessels, experimentally demonstrated by FMD (as detailed in Chapter 1.4.1), is mediated by increased synthesis and release of NO from the endothelium and is reduced by inhibition of nitric oxide synthase by NG-monomethyl-L-arginine (Joannides, 1995).

In the peripheral microcirculation, endothelial function can be assessed non-invasively by measuring blood flow by Doppler velocimetry coupled with direct current iontophoresis of endothelium-dependent vasoactive substances such as ACh. Akin to FMD, ACh-induced vasodilation reflects the release of NO from the endothelium, with

a possible contribution from prostagiandin production (Richards, 1990). The effects of iontophoretically applied ACh are restricted to the cutaneous microvasculature and do not appear to involve sensory nerve activation (Morris, 1996) or activation of nicotinic receptors (Komesaroff, 1998).

The concept that sex hormones may regulate vascular function via effects on the endothelial cell layer has increasingly gained strength as virtually all the traditional coronary risk factors are associated with endothelial dysfunction (Celermajer, 1996; Clarkson, 1997). *In vitro* studies showing that estrogens promote the synthesis of eNOS (Wellman, 1996; Nuedling, 1999) have been complemented by others demonstrating increases in NO secretion following estrogen administration *in vivo* (Gilligan, 1994; White, 1997). Estrogen has also been shown to enhance endothelial cell attachment and proliferation (Morales, 1995).

There is also some evidence that androgens may exert beneficial effects on endothelial function, although results are conflicting. *In vitro* studies have demonstrated that dihydrotestosterone may promote endothelial cell proliferation (Somjen, 1998). In addition, it has been shown that testosterone enhances endothelium-dependent vasodilation in coronary conductance and resistance arteries (Chou, 1996) and in men with coronary artery disease (Ong, 2000). Conversely, other studies have suggested that endothelial function in adult men is enhanced by androgen withdrawal (Herman, 1997).

It has been reported that DHEA may exert direct atheroprotective effects on the vasculature, possibly due in part to the inhibition of atherosclerotic progression and of

smooth muscle and fibroblast cell proliferation (Chapter 1.7.1.6). Previous studies in this thesis have identified potentially atheroprotective effects of DHEA *in vitro* via inhibition of VSMC proliferation and accentuation of endothelial cell proliferation by mechanisms independent of AR and ER (Chapters 2 and 3). Furthermore, we have established that DHEA administration to postmenopausal women is associated with reduced arterial stiffness, possibly due to structural or hemodynamic changes in the arterial wall (Chapter 4).

Although there is limited information on the effects of DHEA on endothelial function *in vivo*, we have shown that DHEA increases the synthesis of eNOS in a dose and time dependent manner (Chapter 3C). This observation raises the possibility that DHEA may alter vascular tone by the restoration of endothelium-dependent vasodilation.

Aims of Study

The current study examined the effects of DHEA on endothelial function *in vivo*. A double-blind, placebo controlled design was employed to investigate the effects of oral DHEA supplementation on endothelium-dependent and -independent vasodilation in peripheral medium sized arteries and subcutaneous microvascular resistance arterioles in of healthy postmenopausal women.

5.2 METHODS

5.2.1 Subjects

As part of the same study described in Chapter 4, an investigation of the effects of DHEA on endothelial function was carried out using the same subjects as previously reported (Chapter 4.2.1). Thirty-six healthy postmenopausal volunteer subjects were recruited by advertisement from the general community previously described (Chapter 4.2.1). All subjects gave written informed consent for their participation in the study, which was approved by the Alfred Hospital Ethics Review Committee. Prior to beginning the study, subjects underwent a screening process to ensure their suitability for participation.

5.2.2 Study Design, Protocol and Pharmacological Interventions

A longitudinal, double-blind, placebo controlled study was used to investigate the effects of three-months DHEA administration on endothelial function. The study design, protocol and pharmacological interventions are detailed in chapter 4.2.2.

5.2.3 Procedures

Resting brachial artery pressure and heart rate were measured with subjects in the supine position for ten minutes (Chapter 4.2.7.1.2). Endothelial function as assessed by endothelium-mediated vasodilation was measured by FMD of the brachial artery in response to reactive hyperemia, and laser Doppler velocimetry (LDV) with direct current iontophoresis of ACh as described below. Blood samples were taken for the

measurement of plasma levels of DHEA, estradiol, estrone and testosterone (Chapter 4.2.6), of total LDL and HDL cholesterol, and of triglycerides (Chapter 4.2.5).

5.2.4 Measures of Endothelial Function

5.2.4.1 Flow Mediated Dilation (FMD)

Brachial artery FMD, a well-established non-invasive procedure reflecting systemic endothelial function, uses high resolution ultrasound to measure changes in arterial diameter in response to reactive hyperemia.

The subject lies supine for the duration of the procedure. Electrocardiogram (ECG) leads attached to the ultrasound machine (Powervision 7000, Toshiba, Japan) are adhered to both wrists and on the left side of the subject's abdomen to record an ECG and to measure the subject's heart rate. A sphygmomanometer cuff is placed on the upper right forearm of the subject distal to the artery and covering the point of bifurcation of the brachial artery.

Following a 15-minute rest period, a high frequency (7.5MHz) linear array transducer is used to obtain a longitudinal image of the artery. Brachial artery diameter is measured from B mode ultrasound 5-7cm above the antecubital crease when the clearest image of the anterior and posterior intimal layers is obtained. When optimum clarity of the lumen to vessel interface is achieved, an anatomical marker on the image is identified to ensure accuracy and proximity of repeated measures of the same section of the artery (Figure 5.1 - A). The image together with a simultaneous ECG is recorded onto high quality standard videocassettes with a video recorder (Sony, model 9500 MDP, Japan) for later

analysis. Brachial artery blood pressure and heart rate are measured at 3-minute intervals by a Dinamap vital signs monitor (1846SX, Critikon, Florida, USA).

Reactive hyperemia was induced by prolonged inflation and abrupt deflation of the sphygmomanometer cuff. The cuff was inflated to a pressure of 250 mmHg for a period of 4.5 minutes in accordance with established protocols, which have shown a maximum dilatory response after 4.5 minutes, whereas a shorter time produces a reduced response and longer time periods no increase (Celermajer, 1992; Leeson, 1997).

Reactive hyperemia-induced dilation of the artery was monitored continuously for 2-3 minutes post cuff deflation. The subject was then allowed to rest for ten minutes to ensure vessel recovery and a final scan was performed over the same area. Figure 5.1 - B demonstrates the reactive hyperemia-dilatory response of the brachial artery compared with baseline conditions.

Endothelium-independent dilation of the brachial artery was assessed by administration of sublingual glyceryl trinitrate (300 μg), an exogenous source of nitric oxide. Scans over the same area of the artery were obtained at baseline and during the vasodilatory response.

Analysis of the ultrasound recordings was performed on a Toshiba 380A SSA ultrasound machine. Brachial artery diameter was measured between the anterior and posterior tunica intima at a fixed distance from the chosen structural marker. Measurements were taken as average artery diameter at peak systole of three consecutive cardiac cycles (as

judged by the ECG trace), at baseline and during FMD, and the percentage increase in FMD was then calculated.

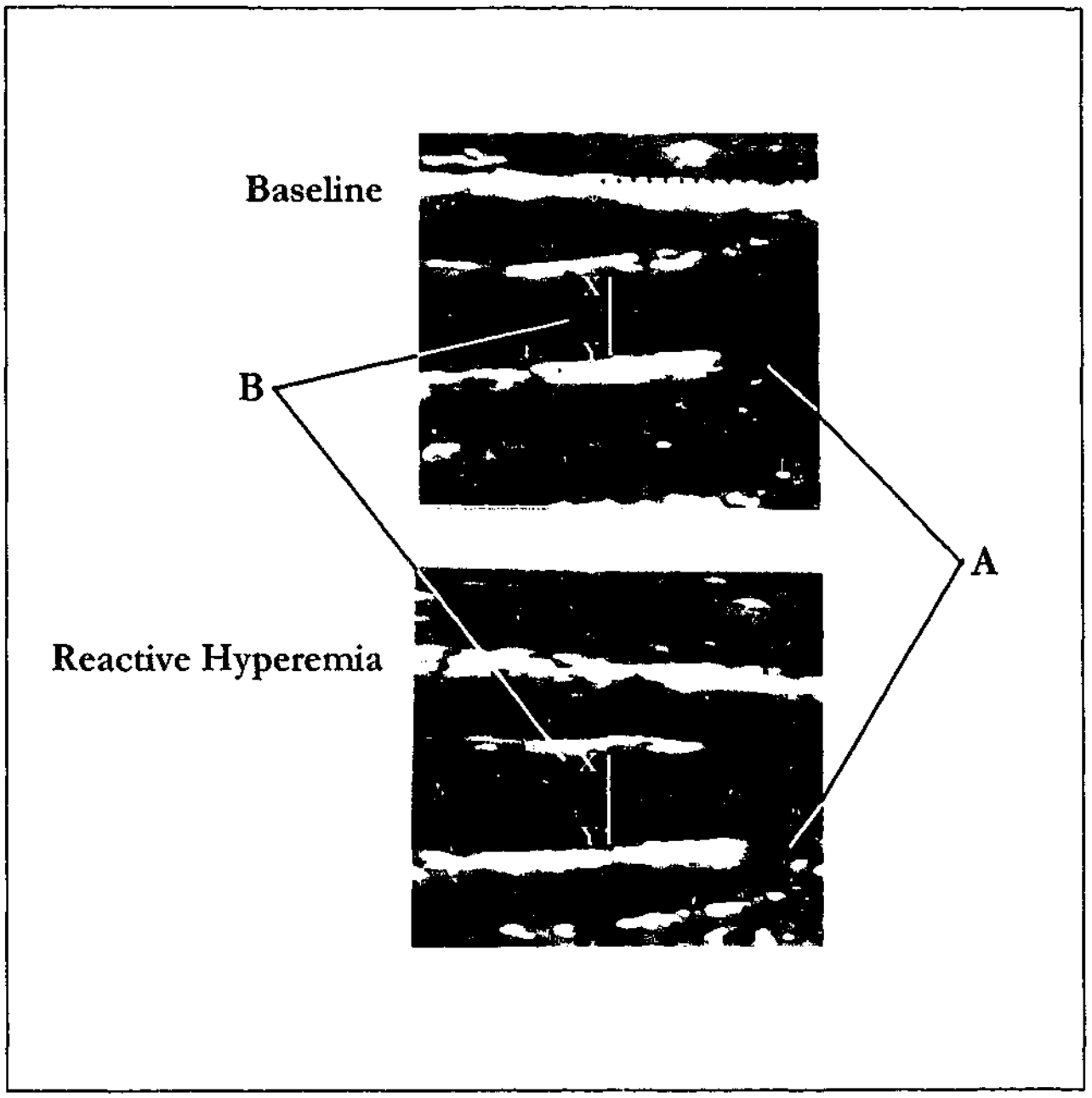


Figure 5.1 Ultrasound Images of the Brachial Artery at Baseline and During Reactive Hyperemia

A Branching vein used as an anatomical structural marker to assess position of brachial artery diameter measurement.

B Measurement of brachial artery diameter at baseline and during reactive hyperemia (distance between X and Y).

5.2.4.2 Laser Doppler Velocimetry (LDV) with Direct Current Iontophoresis

Subcutaneous endothelium-mediated vascular reactivity was measured by direct current iontophoresis with laser Doppler velocimetry (LDV). Erythrocyte flux was measured by a dual channel Moor DT4 laser Doppler flowmeter (Moor Instruments, England), which uses a helium-neon infrared light from a low power laser (wave length of 632.8 nm) via a fibre optic probe to measure change in frequency shift produced by the scatter of photons from moving erythrocytes 1-2 mm below the surface of the skin. The magnitude of the Doppler shift is directly proportional to erythrocyte flux (Westerman, 1988).

With the subject comfortably seated, the right arm is extended across the bed and positioned in a holding apparatus with the anterior surface facing the flowmeter (Figure 5.2 - A). Eight specially designed polyvinyl chloride (PVC) chambers with a central reservoir 12 mm in diameter (0.5 ml volume) were attached to the surface of the forearm by a double-sided adhesive ring. The position of the chambers was chosen to avoid any obvious broken skin or hair growth (Figure 5.2 - B). An indifferent electrode with a soaked tissue underneath was placed on the anterior surface of the wrist to act as an earth (Figure 5.2 - C).

Blood flow was returned to and maintained at basal levels in the forearm vasculature by sustaining the subject in this position for fifteen minutes prior to and for the duration of the procedure. Assessment of erythrocyte flux was recorded on a computer using the Moor Instruments laser Doppler perfusion measurement package V3.01, with each PVC chamber scanned consecutively 25 times over a period of approximately 6 minutes

incorporating 2 baseline scans, 30 seconds of iontophoresis, and the resulting vasodilatory response.

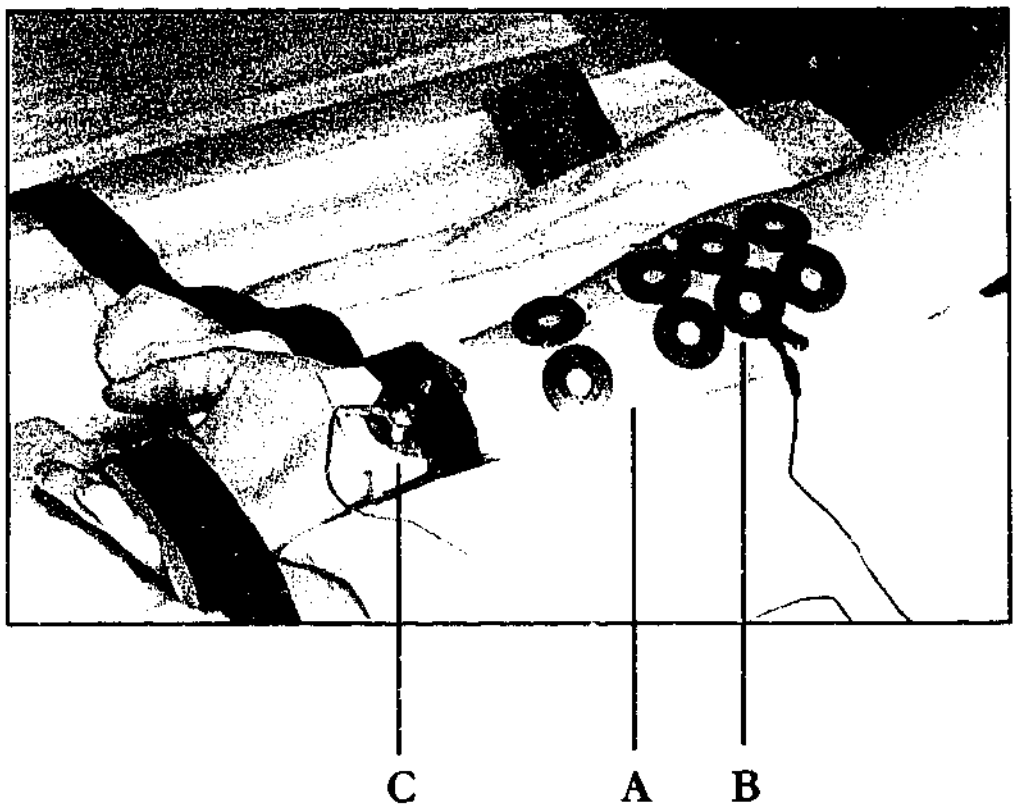


Figure 5.2 Measurement of Subcutaneous vascular reactivity by LDV with Direct Current Iontophoresis

A With the subject comfortably seated, the right arm is extended across the bed and positioned in a holding apparatus with the anterior surface facing the flowmeter.

B PVC chambers with a central reservoir 12 mm in diameter (0.5 ml volume) are attached to the surface of the forearm. The position of the chambers was chosen to avoid broken skin or hair growth.

C An indifferent electrode with a soaked tissue underneath was placed on the anterior surface of the wrist to act as an earth.

Endothelium-dependent vascular reactivity was assessed by the iontophoresis of ACh (BDH Chemicals, UK). The nitric oxide donor sodium nitroprusside (SNP) (David Bull Laboratories, Australia) was iontophoresed as an endothelium-independent vasodilator control. Solutions of ACh and SNP (10 mg/ml) were prepared in a methyl cellulose gel (10% w/v) (Sigma) at least 24 hours before being placed in the chamber reservoir.

The circuit was completed by placing a metal clip onto a platinum wire, which lined the interior diameter surface of the chamber reservoir at the chamber/skin surface interface (see Fig 5.2). An iontophoresis controller was used to provide a direct current for drug iontophoresis. To assess endothelium-dependent vasodilator dose responsiveness, ACh (10mg/mL) iontophoresis used anodal current of 0.1 mA for 30 seconds. SNP (10mg/ml) was iontophoresed by a cathodal current of 0.1 mA for 30 seconds. The coefficient of variation for blood flow assessed by this method of measuring subcutaneous vascular reactivity is 0.45 ± 0.05.

Blood perfusion, measured as a frequency shift by the laser Doppler flowmeter, was computer analyzed by the Moor Instruments laser Doppler perfusion measurement package V3.01 and translated into an image. The images are displayed with colours representing the degree of blood flux on a scale of 250 units (Figure 5.3). The responses were quantified by measuring the area under the curve of each response using Microsoft Excel (Microsoft Office 2000). Data are represented as arbitrary perfusion units.

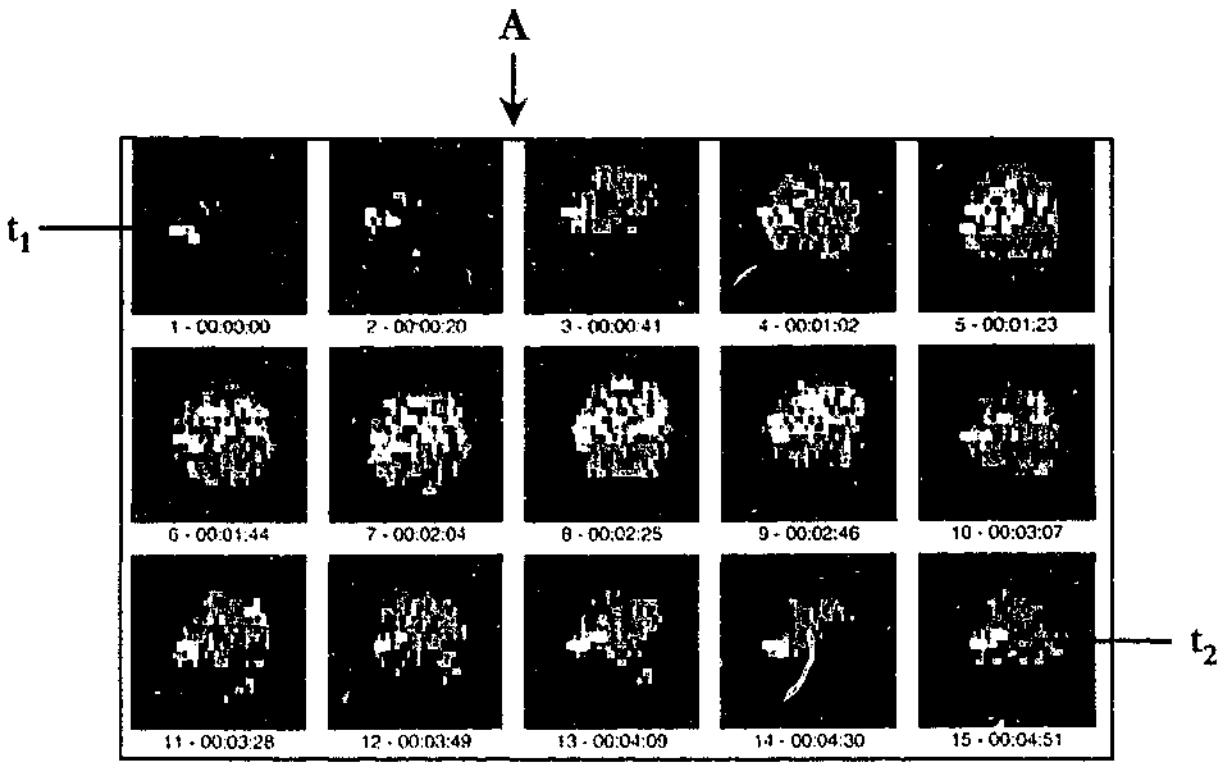


Figure 5.3 Blood Perfusion as Measured by the Laser Doppler Flowmeter
Blood perfusion was computer analyzed by the Moor Instruments laser Doppler flowmeter and translated into an image. The image is displayed as a series of smaller images with colors representing the degree of blood flux over a period of time ($t_1 - t_2$).

A Time of iontophoresis of either ACh or SNP following two baseline scans.

5.2.5 Statistical analysis

All data are presented as mean ± SEM. Statistical analysis between two observations was by Student's t-test and in multiple comparisons by analysis of variance. The null hypothesis was rejected at $p>0.05$.

5.3 RESULTS

5.3.1 Subject characteristics and effects of DHEA on hormone levels and lipid profile

Table 4.1 shows that subjects in both treatment groups were well matched for the baseline characteristics. There were no significant differences in any of the variables measured. Table 4.2 shows the effects of three months of DHEA treatment on DHEA, estradiol, estrone and testosterone levels, and serum lipid profile.

5.3.2 Effects of DHEA on FMD of the Brachial Artery in Response to Reactive Hyperemia

There were no differences in baseline measures of %FMD between the two treatment groups (8.4 ± 0.7 and 10.7 ± 1.1% for DHEA and placebo respectively). Following 12 weeks of DHEA administration, brachial artery FMD increased significantly to 14.5 ± 1.1%, $p<0.05$. There were no changes in FMD following 12 weeks of placebo administration. Fig 5.4A illustrates the effects of 12-week supplementation of DHEA on %FMD of the brachial artery compared to those of placebo.

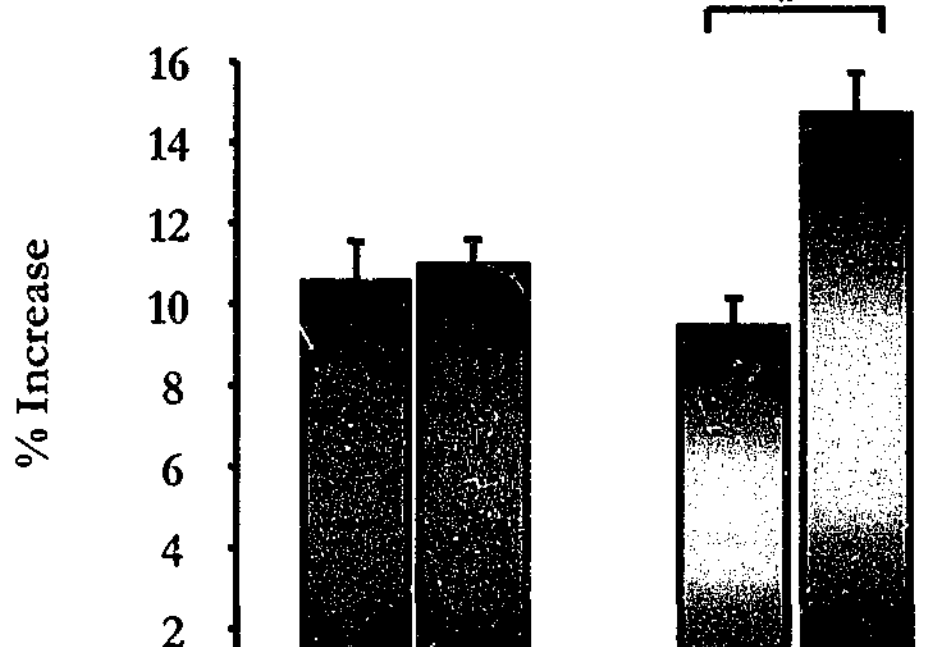
Sublingual glyceryl trinitrate (GTN)-induced endothelium-independent brachial artery vasodilation was unaltered between measurement time points in both DHEA and placebo treatment groups (18.5 ± 1.1% compared to 16.4 ± 1.2% for DHEA and 18.4 ± 1.4% compared to 19.2 ± 1.3% for placebo treatment, $p= 0.2$ and 0.7 respectively) (Fig 5.4B). In all women, resting brachial artery diameter did not change throughout the duration of the study.

5.3.3 Effects of DHEA on LDV with direct current iontophoresis

Endothelium-dependent subcutaneous blood flow in response to iontophoresed ACh was significantly enhanced following 12 weeks of DHEA administration compared with baseline values (16 ± 2AU compared to 32 ± 4AU, $p<0.05$). There were no differences observed in ACh-induced dilation between measurement time points in the placebo treated group (18 ± 2AU compared to 20 ± 2AU). Figure 5.5A illustrates the effects of DHEA and placebo on endothelium-dependent vasodilation in response to ACh.

Endothelium-independent subcutaneous blood flux in response to SNP ionotophoresis was unaltered between the measurement time points in both DHEA and placebo treatment groups (2519 ± 316AU compared to 2755 ± 442AU for DHEA and 2390 ± 308AU compared to 2763 ± 564AU for placebo treatment) (Fig 5.5B).

A.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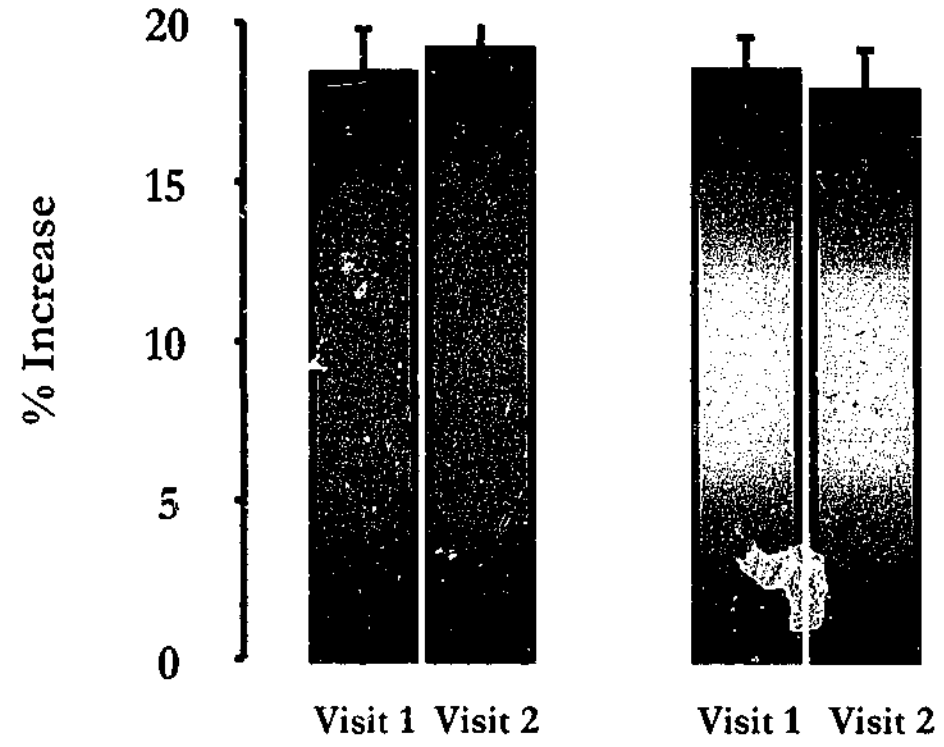
B.


20
15
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5
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% Increase
Visit 1 Visit 2
Visit 1 Visit 2
Placebo
DHEA

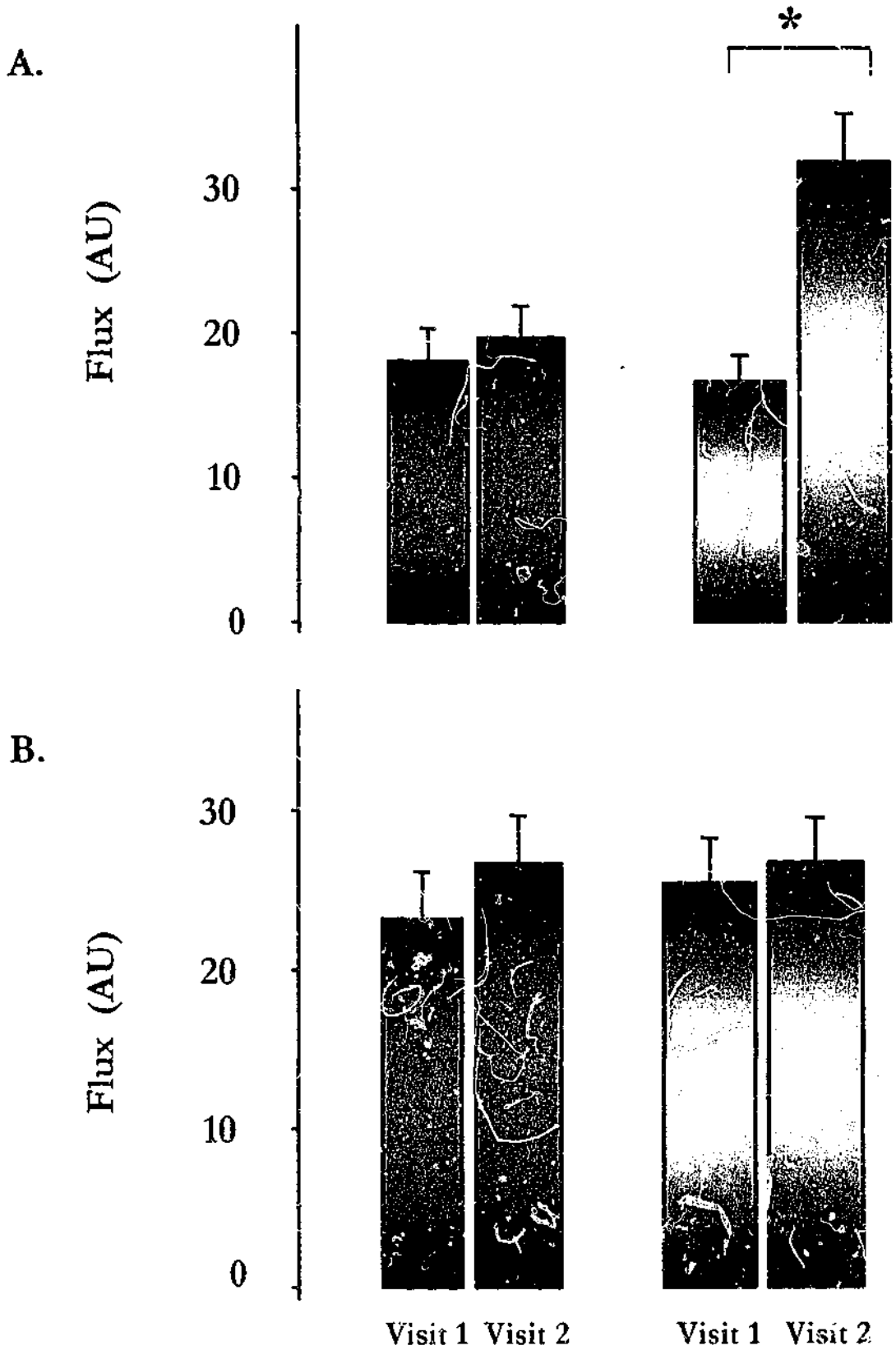
Figure 5.4 Effects of DHEA on %FMD in Healthy Postmenopausal Women

A. FMD of the brachial artery with reactive hyperemia is significantly increased following three months of DHEA administration (8.4 ± 0.7 to 14.5 ± 1.1%, $p<0.05$). There were no changes in FMD following placebo treatment (10.8 ± 1.1 to 10.9 ± 0.6%).

B. Sublingual glyceryl trinitrate (GTN)-induced endothelium-independent vasodilation was unaltered between measurement time points in both DHEA and placebo treatment groups (18.5 ± 1.1% compared to 16.4 ± 1.2% for DHEA and 18.4 ± 1.4% compared to 19.2 ± 1.3% for placebo treatment, $p= 0.2$ and 0.7 respectively).

Data are presented as mean percentage FMD ± SEM.

* denotes a significant difference between measurement time points.


A.
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Flux (AU)
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20
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Visit 1 Visit 2
Placebo
Visit 1 Visit 2
DHEA

Figure 5.5 Effects of DHEA on Cutaneous Vascular Reactivity in Healthy Postmenopausal Women

A. Endothelium-dependent subcutaneous blood flow in response to iontophoresed ACh was significantly increased following DHEA administration compared with baseline values (16 ± 2AU compared to 32 ± 3AU, $p<0.05$) . There were no differences observed in ACh-induced dilation between measurement time points in the placebo treated group (18 ± 2AU compared to 20 ± 2AU).

B. Endothelium-independent subcutaneous blood flux in response to SNP ionotophoresis was unaltered between the measurement time points in both DHEA and placebo treatment groups (2519 ± 315AU compared to 2754 ± 442AU for DHEA and 2390 ± 308AU compared to 2762 ± 564AU for placebo treatment, $p= 0.67$ and 0.2 respectively).

Data are presented as mean arbitrary flux units (AU) ± SEM.
* denotes a significant difference between measurement time points.

5.4 DISCUSSION

This is the first study to investigate the effects of DHEA administration on endothelial function in healthy postmenopausal women. The current study demonstrated that three-month oral DHEA supplementation in postmenopausal women increases endothelium-mediated dilation, as measured by FMD of the brachial artery in response to reactive hyperemia and LDV with direct current iontophoresis of ACh, compared to placebo treatment. These observations were found to be independent of mean arterial pressure and heart rate as these parameters did not change significantly throughout the study.

An increasing body of evidence supports the fundamental role for the endothelium in the regulation of vascular function in health and disease. Under normal physiological conditions, the vascular endothelium provides a highly thromboresistant surface to flowing blood (Schafer, 1997), prevents leukocyte and platelet adhesion, and inhibits proliferation of vascular smooth muscle cells (Chapter 1.3.1). However, in states of endothelial dysfunction, such as atherosclerosis, in which concentrations of bioactive NO are reduced, the relatively unopposed actions of vasoconstrictor substances, such as ET-1, promote vasoconstriction and smooth muscle proliferation (Lopez, 1990) (Chapter 1.3.1).

An important and rapid physiological adaptation to changes in humoral or hemodynamic conditions in conduit and resistance arteries is the regulation of arterial diameter. Increases in arterial blood flow and interactions between factors such as ACh and receptors on the endothelial surface, result in endothelium-dependent relaxation of

smooth muscle tone and subsequent arterial dilation. The mechanisms of ACh and shear stress-induced arterial dilation involve the release of vasoactive factors from the endothelium, the principal components of which are NO and other closely related nitroderivatives (Palmer, 1987; Palmer, 1988; Moncada, 1991). Prostacyclin has also been shown to be involved in shear stress induced dilation (Frangos, 1985).

Increasing data suggest that sex hormones are important contributors to the maintenance of vascular function via direct effects on the endothelium. Estrogen supplementation has been shown to increase peripheral perfusion and decrease impedance to blood flow in postmenopausal women (Gangar, 1993; Sudhir, 1995; Volterrani, 1995; Sanada, 2001), possibly by stimulating nitric oxide release (Wren, 1992; Gilligan, 1995; Sudhir, 1996; Sanada, 2001). Indeed, NO-dependent dilation has been shown to be impaired in an adult male with estrogen deficiency (Sudhir, 1997). Further, estrogen has been shown to increase both ACh-induced (Komesaroff, 1998; New, 1999) and FMD-mediated endothelium-dependent dilation (Perregaux, 1999).

Unlike estrogens, the effect of androgens on endothelium-dependent and independent vasodilation has not been well defined. A recent study by Worboys et al. has shown that testosterone administration increases endothelium-dependent vasodilation in coronary circulation (Worboys, 2001). In addition, other studies have shown attenuation of testosterone-induced vasorelaxation after L-NAME pretreatment at both the epicardial and microvascular level (Chou, 1996), suggesting that testosterone influences vascular reactivity via endothelium-dependent mechanisms. However, *in vitro* studies have shown

that testosterone relaxes rabbit coronary arteries acutely via mechanisms which appear to be endothelium-independent (Yue, 1995).

We have previously reported that DHEA increases endothelial cell proliferation (Chapter 3A) and stimulates eNOS production (Chapter 3C), suggesting a potentially beneficial role for DHEA in regulating endothelial function. In accordance with these *in vitro* findings, the current study has demonstrated that DHEA increases FMD-induced vasorelaxation as well as microcirculatory vascular reactivity via endothelium-dependent mechanisms. Therefore, these results suggest that DHEA may afford atheroprotection, at least in part, via increases in endothelium-dependent vasodilation in the peripheral vasculature.

As previously argued in relation to SAC (Chapter 4), we propose that the increases in endothelium-dependent vascular reactivity observed reflect direct DHEA interactions with the vessel wall rather than DHEA conversion to androgens and/or estrogens. While the vasodilatory properties of estradiol are well documented, there have been no studies to substantiate the potential capacity for estrone independent of estradiol in mediating vasodilation. Furthermore, the testosterone concentrations reported to induce significant vasodilation in the coronary vasculature are in excess of 100 nmol/L (Chou, 1996), levels substantially higher than those achieved in the current study or those found in healthy male volunteers (21±1 nmol/L) (Cantrill , 1984). Therefore, while the present study suggests that changing the hormonal milieu by oral administration of DHEA is associated with changes in large vessel and microvascular vascular reactivity, further

investigation is warranted to elucidate the mechanism of DHEA action in promoting endothelium-mediated vasodilation.

5.5 CONCLUSIONS

The present study provides direct evidence in support of a potential cardioprotective role of DHEA in healthy postmenopausal women, mediated in part, via effects on endothelial function in resistance arteries and the peripheral subcutaneous vasculature. We have demonstrated that DHEA significantly augments endothelium-mediated vasodilation of the brachial artery, as measured by FMD in response to reactive hyperemia, and endothelium-dependent subcutaneous vascular reactivity, as measured by LDV with direct current iontophoresis of ACh. The findings of the current study implicate the peripheral vasculature as a potential site for DHEA action and extend our current understanding of the potential mechanisms by which DHEA may contribute to the lowering of the incidence of cardiovascular disease.

Chapter 6

General Discussion

The rationale underlying the studies conducted in this project relates to the fact that DHEA has been implicated in the possible protection against CVD. However, while the occurrence and clinical manifestation of atherosclerotic disease have been correlated to plasma levels of DHEA, its effects and precise modes of action on the cardiovascular system remain unresolved. In addition, as DHEA is a natural precursor to both androgens and estrogens, the possibility of its having biological actions in its own right has been largely overlooked.

The studies in this dissertation have provided *in vitro* and *in vivo* evidence for a beneficial role for DHEA in cardiovascular physiology. The hypothesis that DHEA may influence the behaviour of smooth muscle and endothelial cells independently of androgen and estrogen receptor-mediated mechanisms has been addressed. In addition, binding studies have confirmed the presence of high affinity DHEA binding sites in both VSMC and endothelial cells. Moreover, some of the subcellular regulatory pathways by which DHEA exerts its effects in these cell types were investigated. *In vivo* approaches have demonstrated that DHEA affects both the central and the peripheral vasculature through its influences on arterial mechanical properties and vascular reactivity.

One of the earliest manifestation of the atherosclerotic disease process is vascular injury, which is characterized by compromised endothelial integrity and increased VSMC proliferation and migration. It is well recognized that sex hormones, and in particular estrogens, can affect VSMC and endothelial cell behavior, and DHEA is a natural precursor to both androgens and estrogens, it was hypothesized that DHEA may exert its actions on the cardiovascular system through similar effects. In accordance with this,

the *in vitro* studies conducted in this project have shown that DHEA may have a beneficial cardiovascular role by inhibiting VSMC proliferation and stimulating endothelial cell angiogenesis. In addition, the observed DHEA effects are mediated specifically via ERK1/2 signaling pathways. Of significant importance are the findings that neither the androgen receptor antagonist flutamide or the estrogen receptor antagonist ICI 182,780 influenced the inhibitory and stimulatory effects of DHEA on VSMC and endothelial cell proliferation respectively, and the demonstration of the presence of DHEA-specific binding sites in these cells. These findings strongly suggest that the mode of DHEA action on cell proliferation is unlike that of either estrogen or testosterone and is dependent on DHEA-receptor complex interactions. However, further studies are required to characterize the molecular structure of the putative DHEA receptor in vascular cells.

The second major focus of this project was to investigate the effects of DHEA administration on large artery mechanical properties and endothelial function of the peripheral vasculature in healthy postmenopausal women. We have demonstrated that large artery stiffness, which is associated with ageing and the development of CHD, is significantly reduced in healthy postmenopausal women taking DHEA treatment, compared to women not taking DHEA supplementation. The reduced arterial stiffness seen in those women taking DHEA was accompanied with lower central systolic and pulse pressures. These findings implicate the central vasculature as a potential target for DHEA-mediated cardioprotective effects.

Compromised endothelial function is associated with the manifestation of many of the known coronary risk factors. Therefore, the findings that DHEA stimulates eNOS expression *in vitro,* taken together with those demonstrating that DHEA intervention significantly affected endothelial function through increased endothelium-dependent vasodilation in postmenopausal women further, support a potential cardioprotective role for DHEA. The actions of DHEA are mediated, at least in part, via effects on endothelial function in resistance arteries and peripheral subcutaneous vasculature, as measured by FMD and LDV with direct current iontophoresis. These findings suggest that the peripheral vasculature may be an additional potential target for DHEA action in the cardiovascular system.

In conclusion, the studies presented in this thesis have shown, by both *in vitro* and *in vivo* approaches, a potentially beneficial role for DHEA in the setting of cardiovascular physiology. These studies also extend our current understanding of the potential mechanisms by which DHEA may contribute to the lowering of cardiovascular disease. In addition, the findings outlined in this dissertation may provide valuable insight regarding the suitability of DHEA administration in humans. However, further research is required to determine whether these beneficial effects are translated into clinical endpoints.

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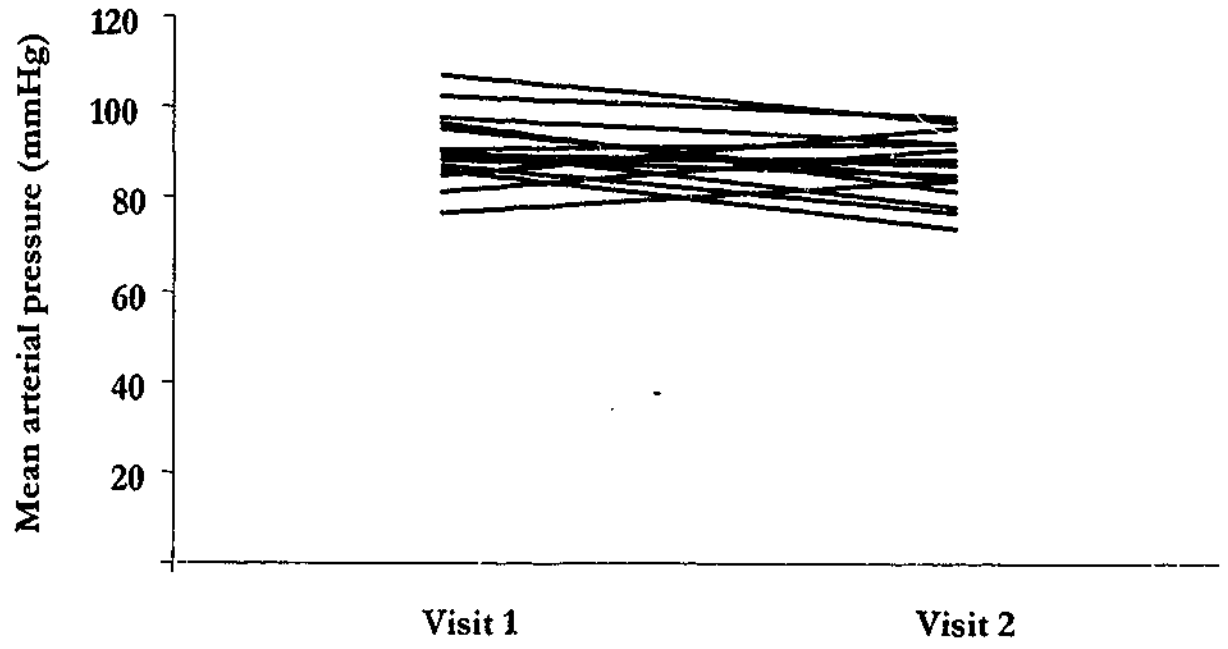
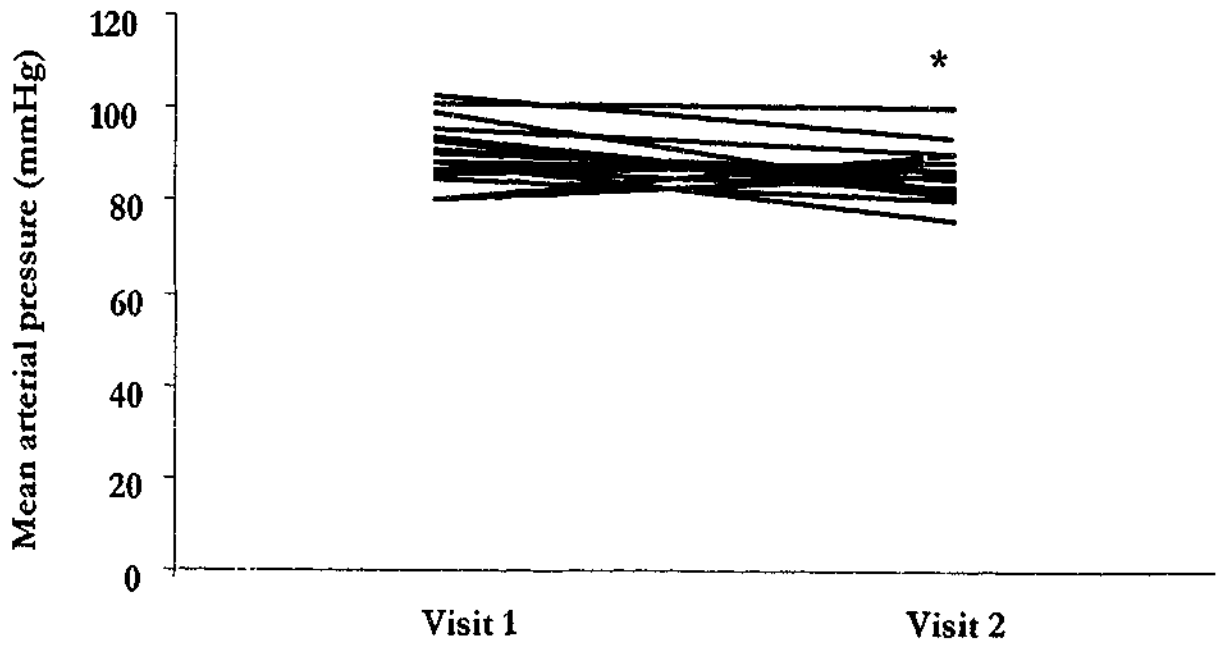
Appendix

A


Mean arterial pressure (mmHg)
120
100
80
60
40
20
Visit 1
Visit 2

B


Mean arterial pressure (mmHg)
120
100
80
60
40
20
0
*
Visit 1
Visit 2

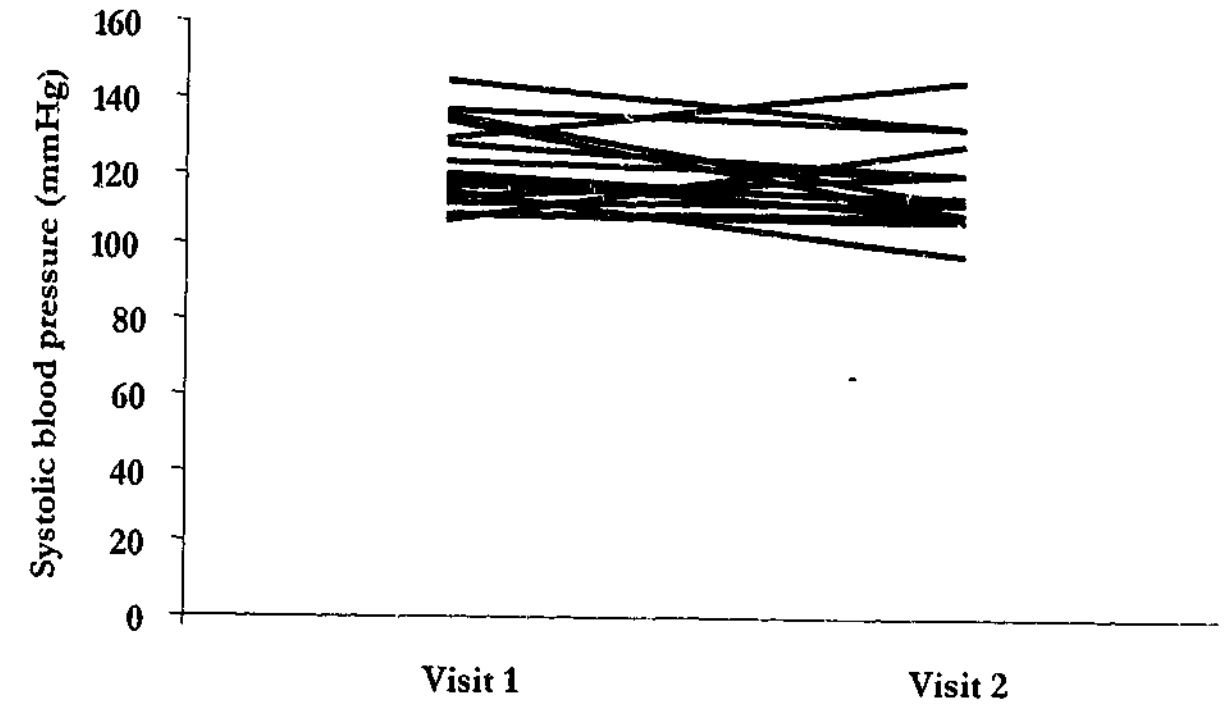
Appendix Figure 1. The Effects of DHEA on Mean Arterial Pressure in Healthy Postmenopausal Women

No significant changes were observed mean arterial pressure (MAP) at baseline compared to 12-week DHEA administration **(A)** (91.3±2.7 and 90.5±2.9 mmHg) as assessed by brachial artery blood pressure measurements. However, there was a significant reduction in MAP in the placebo treatment group **(B)** (90.2±1.7 and 86.4±1.8 mmHg, $p<0.03$) (refer to Table 4.2).

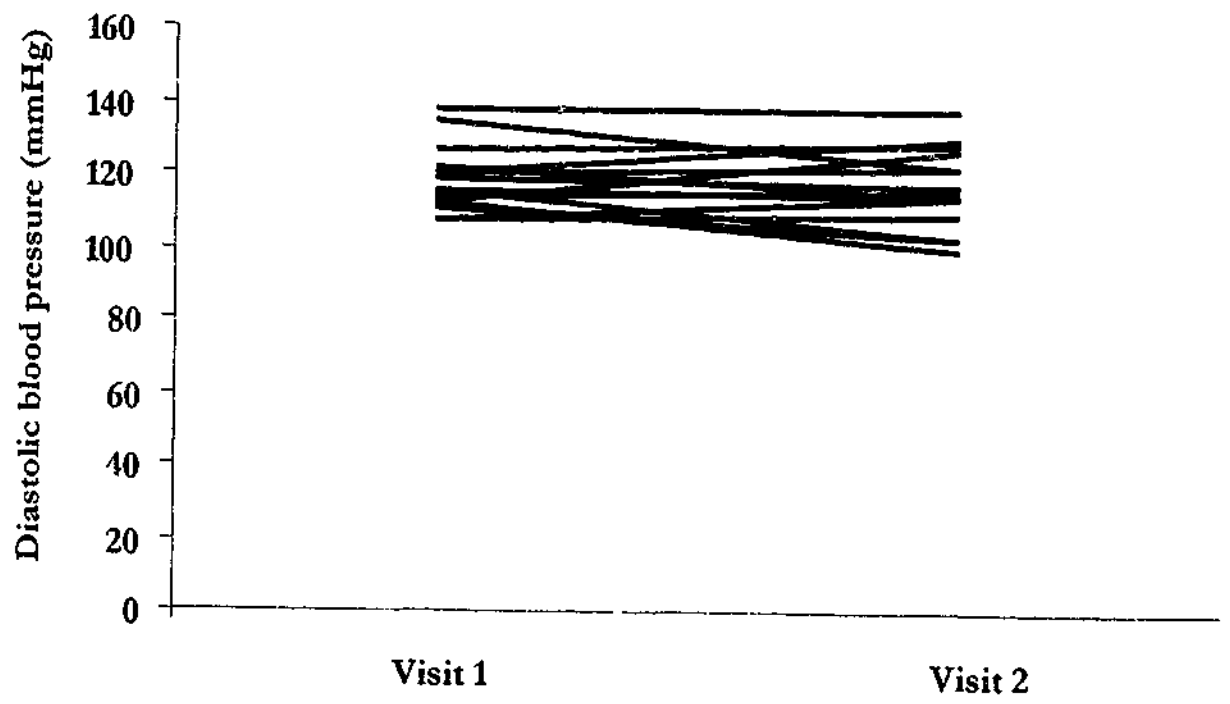
Data are presented as individual patient mean arterial pressures.

* denotes a significant difference in mean MAP ± SEM from baseline

A


Systolic blood pressure (mmHg)
160
140
120
100
80
60
40
20
0
Visit 1
Visit 2

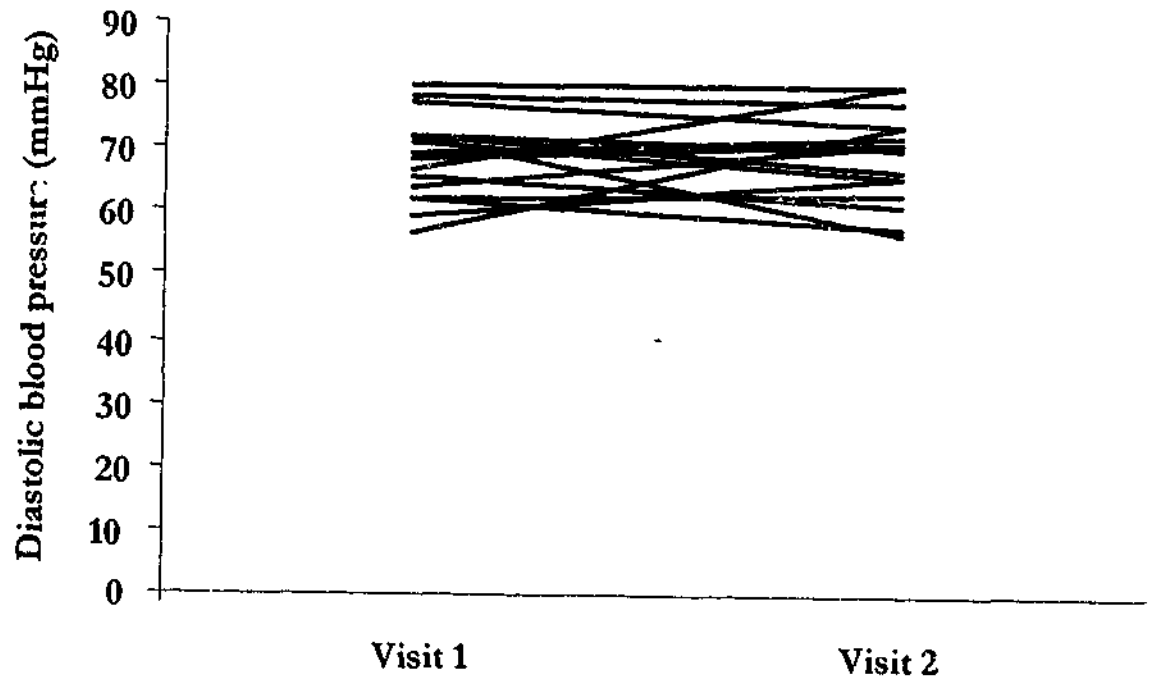
B


Diastolic blood pressure (mmHg)
160
140
120
100
80
60
40
20
0
Visit 1
Visit 2

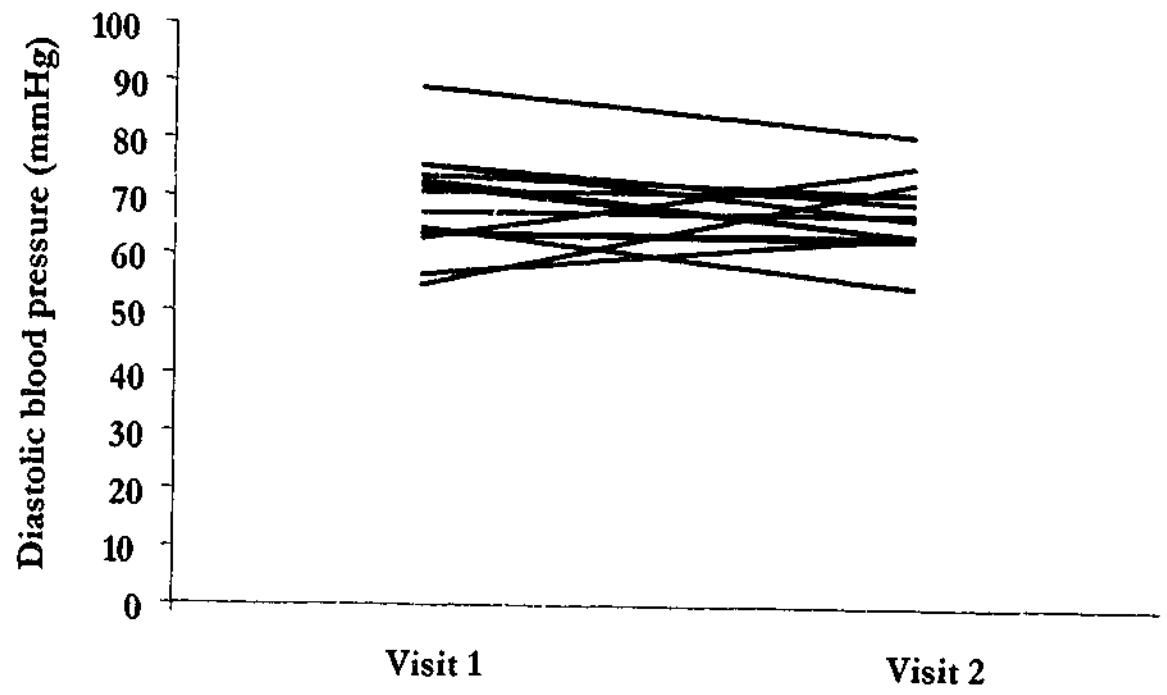
Appendix Figure 2. The Effects of DHEA on Systolic Blood Pressure in Healthy Postmenopausal Women

No significant changes were observed mean systolic blood pressure at baseline compared to 12-week DHEA administration **(A)** (121.1±3.4 and 117.9±3.6 mmHg) as assessed by brachial artery blood pressure measurements. There were no significant changes in mean systolic blood pressure in the placebo treatment group **(B)** (117.9±2.3 and 116.5±2.8 mmHg) (refer to Table 4.2). Data are presented as individual patient systolic blood pressures.

A


Diastolic blood pressure (mmHg)
90
80
70
60
50
40
30
20
10
0
Visit 1
Visit 2

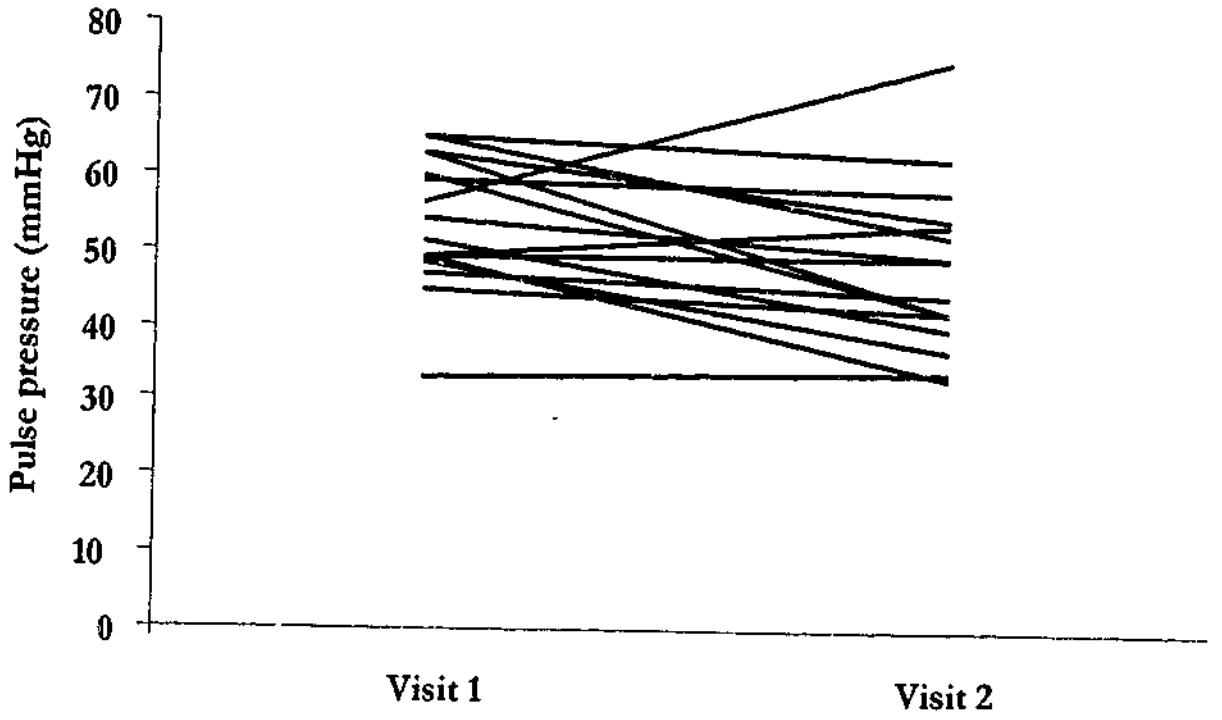
B


Diastolic blood pressure (mmHg)
100
90
80
70
60
50
40
30
20
10
0
Visit 1
Visit 2

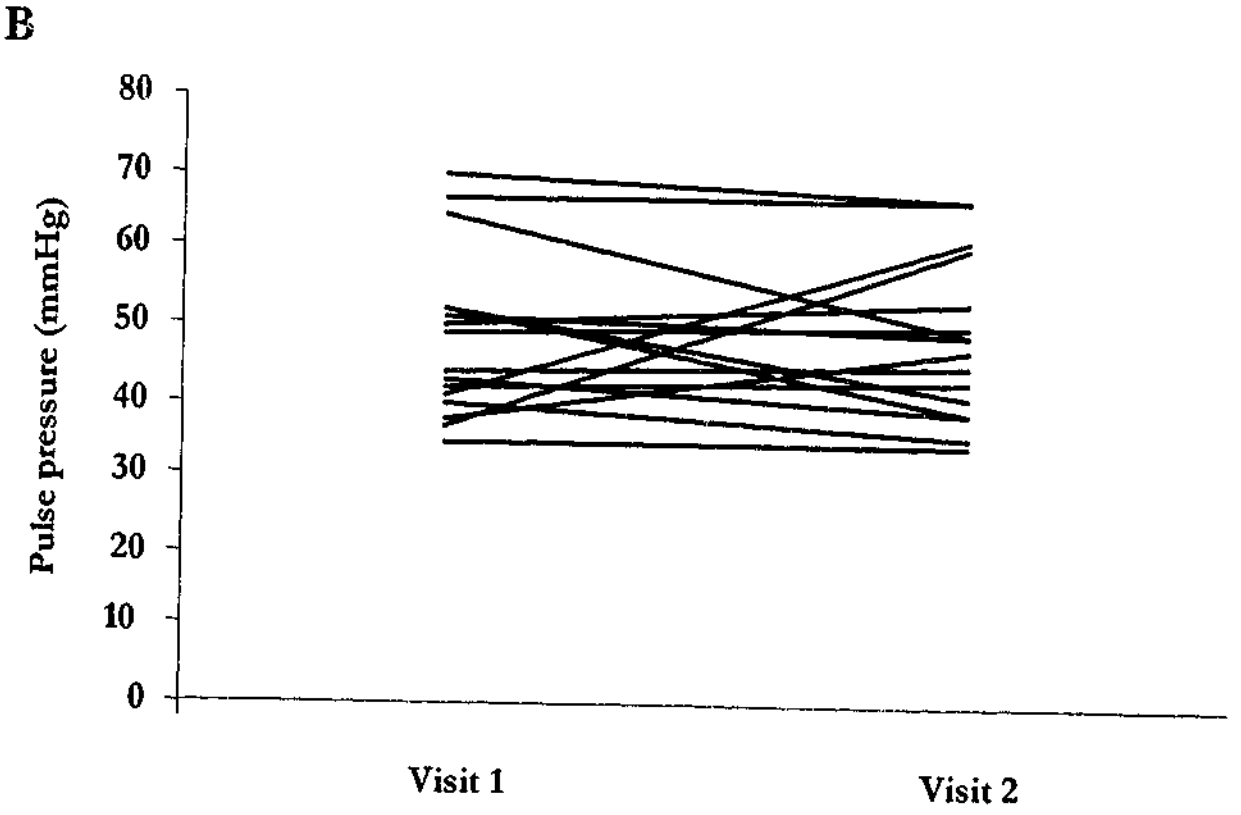
Appendix Figure 3. The Effects of DHEA on Diastolic Blood Pressure in Healthy Postmenopausal Women

No significant changes were observed in mean diastolic blood pressure at baseline compared to 12-week DHEA administration **(A)** (68.6±2.0 and 69.1±2.1 mmHg) as assessed by brachial artery blood pressure measurements. There was no change in mean diastolic blood pressure in the placebo treatment group **(B)** (69.9±2.3 and 68.1±1.6 mmHg) (refer to Table 4.2).
Data are presented as individual patient systolic blood pressures.

A


80
70
60
50
40
30
20
10
0
Pulse pressure (mmHg)
Visit 1
Visit 2

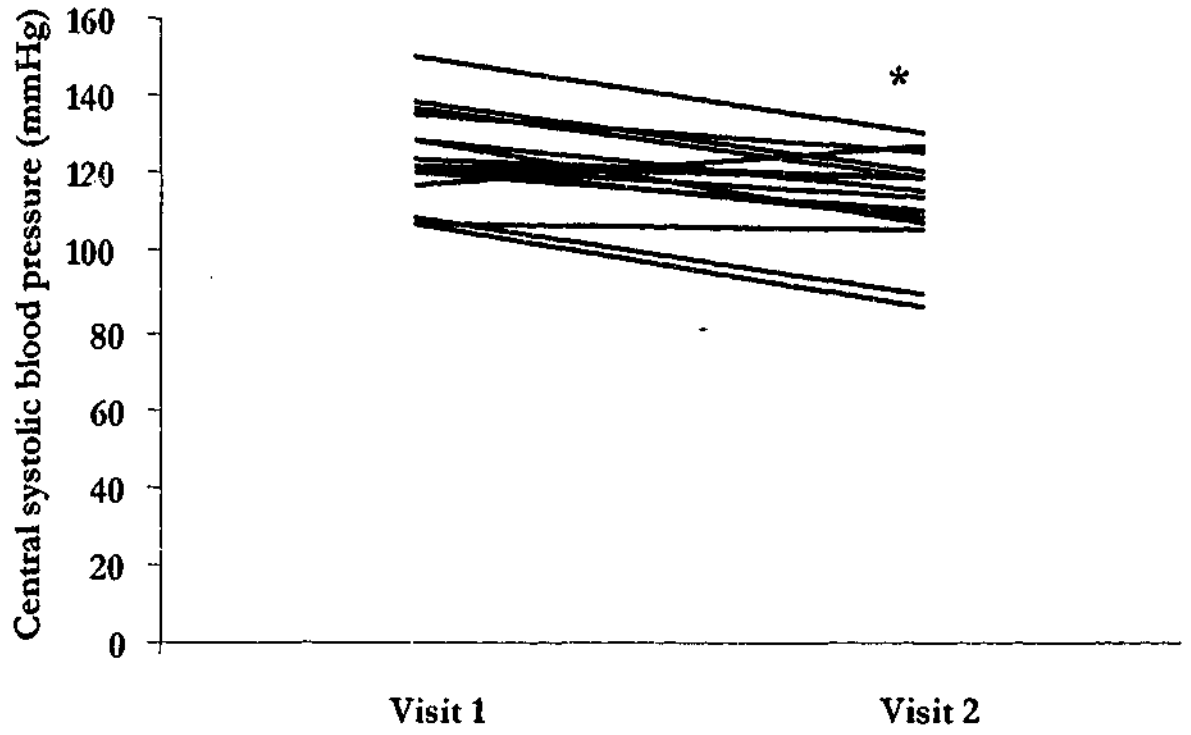
B


80
70
60
50
40
30
20
10
0
Pulse pressure (mmHg)
Visit 1
Visit 2

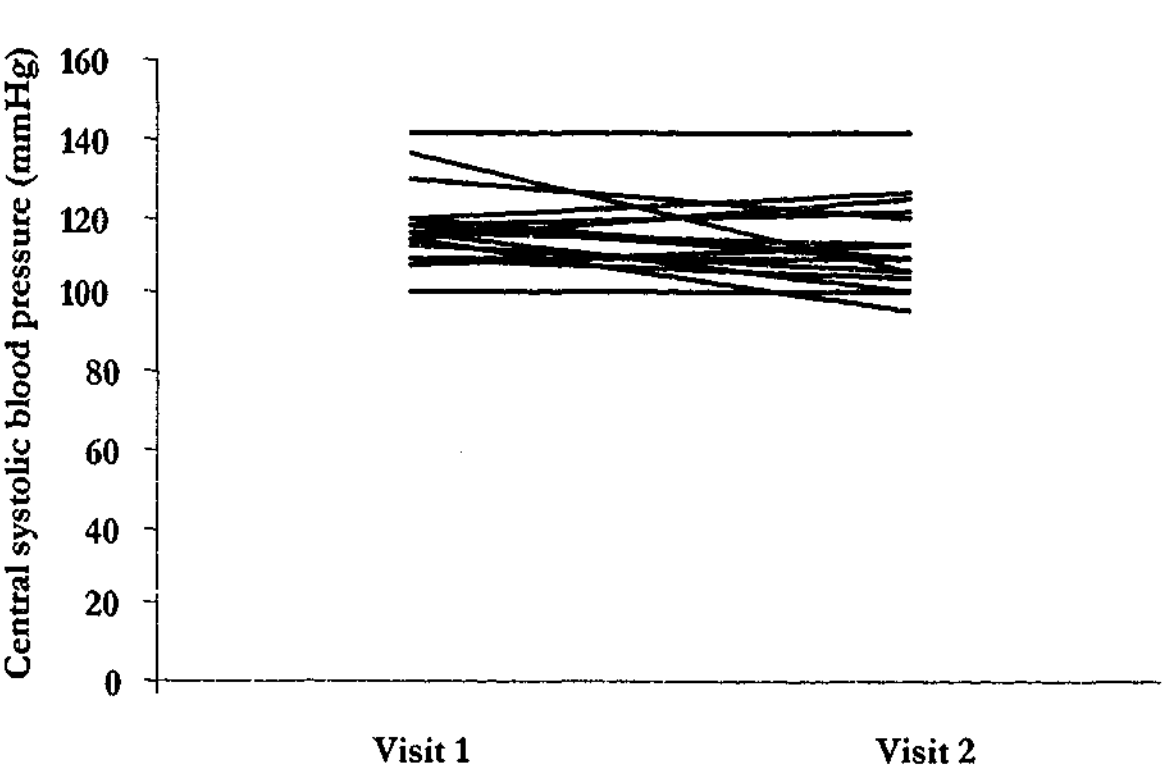
Appendix Figure 4. The Effects of DHEA on Pulse Pressure in Healthy Postmenopausal Women

No significant changes were observed mean pulse blood pressure at baseline compared to 12-week DHEA administration **(A)** (52.5±2.5 and 48.9±3.6 mmHg) as assessed by brachial artery blood pressure measurements. There was no change in mean diastolic blood pressure in the placebo treatment group **(B)** (48±2.9 and 48.4±2.9 mmHg) (refer to Table 4.2).
Data are presented as individual patient pulse pressures.

A


Central systolic blood pressure (mmHg)
160
140
120
100
80
60
40
20
0
*
Visit 1
Visit 2

B


Central systolic blood pressure (mmHg)
160
140
120
100
80
60
40
20
0
Visit 1
Visit 2

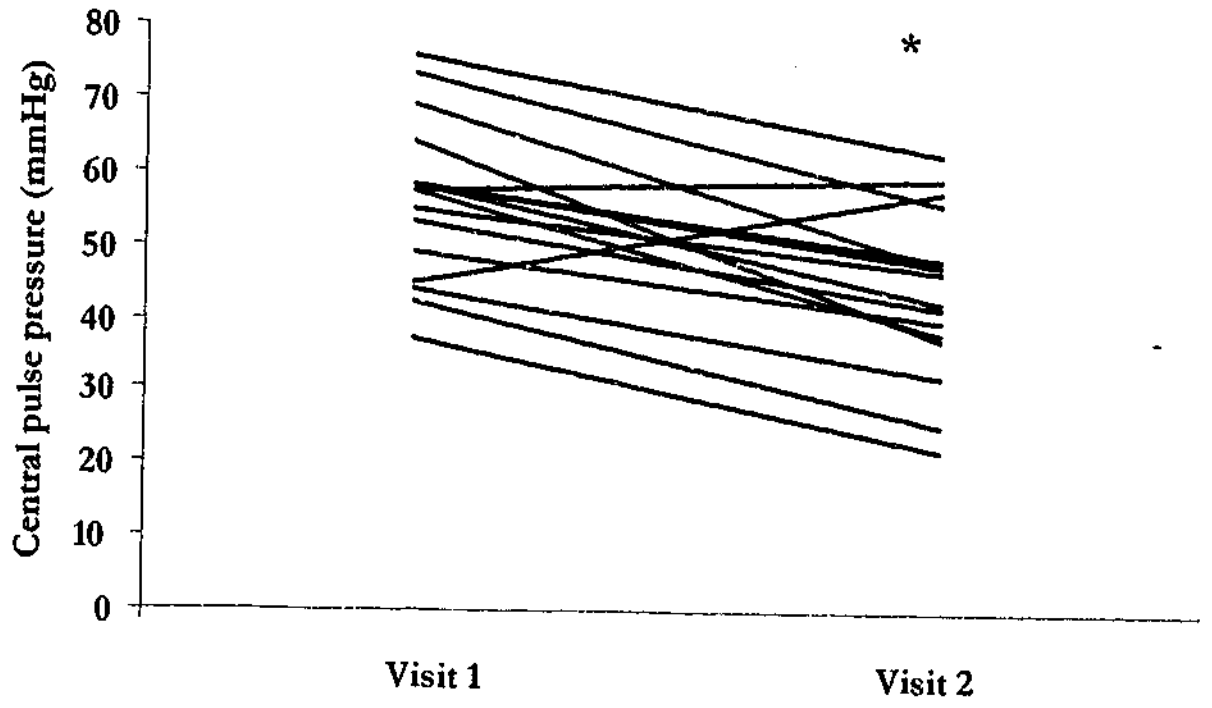
Appendix Figure 4. The Effects of DHEA on Central Systolic Blood Pressure in Healthy Postmenopausal Women

Significant differences were observed in measures of central (carotid) arterial pressures in the DHEA treatment group. Mean values for central systolic blood pressure were significantly decreased **(A)** (124.5±3.6 to 112.8±3.7, p=0.03). There were no changes observed in central systolic blood pressure following placebo treatment **(B)** (117.8±2.9 to 112±3.2) (refer to Table 4.2).

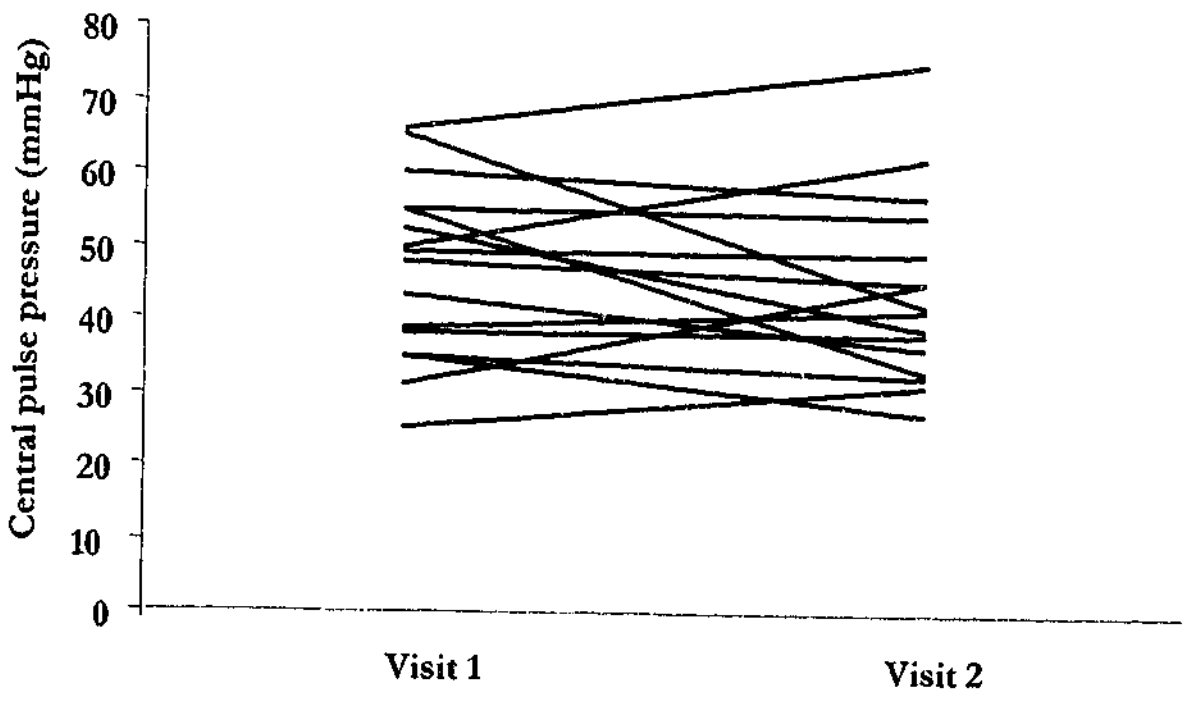
Data are presented as individual patient central systolic blood pressures.

* denotes a significant difference in mean central systolic blood pressure ± SEM, compared to baseline

A


Central pulse pressure (mmHg)
80
70
60
50
40
30
20
10
0
*
Visit 1
Visit 2

B


Central pulse pressure (mmHg)
80
70
60
50
40
30
20
10
0
Visit 1
Visit 2

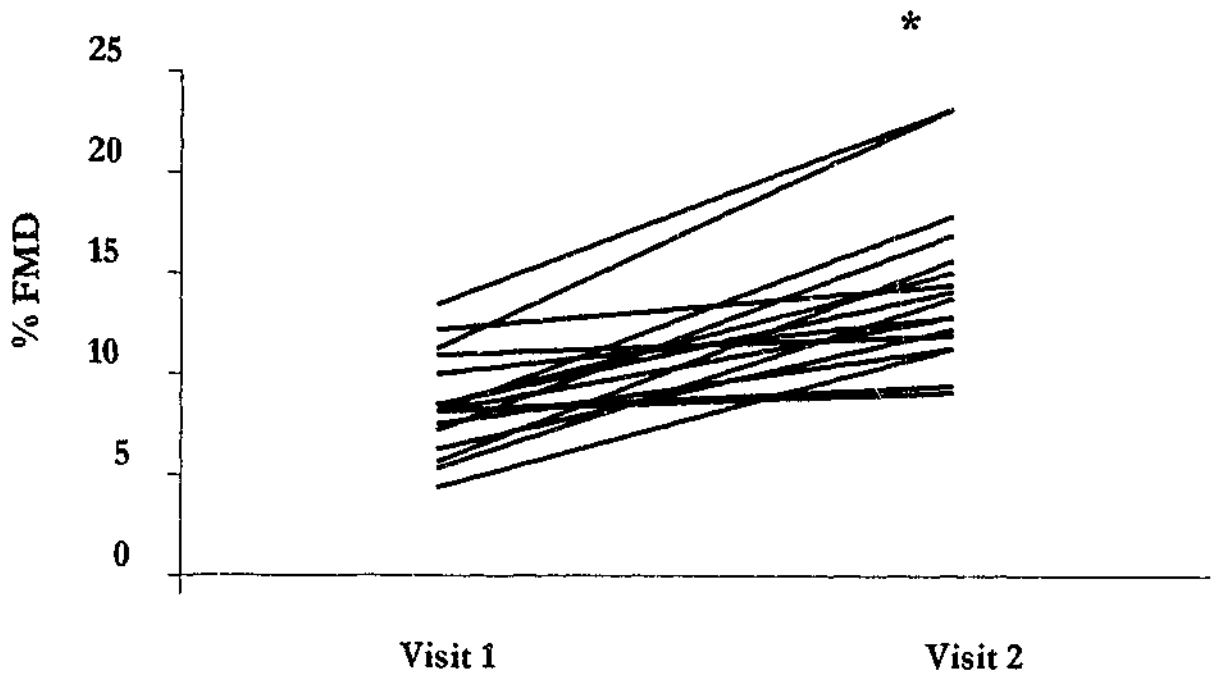
Appendix Figure 4. The Effects of DHEA on Central Pulse Pressure in Healthy Postmenopausal Women

Significant differences were observed in measures of central pulse pressures in the DHEA treatment group. Mean values for central pulse blood pressure were significantly decreased (55.8±3.4 to 43.7±3.6, p=0.02) in the DHEA treatment group **(A)**. There were no changes observed in central pulse pressures following placebo treatment **(B)** (47.9±3.3 to 44.3±3.2) (refer to Table 4.2).

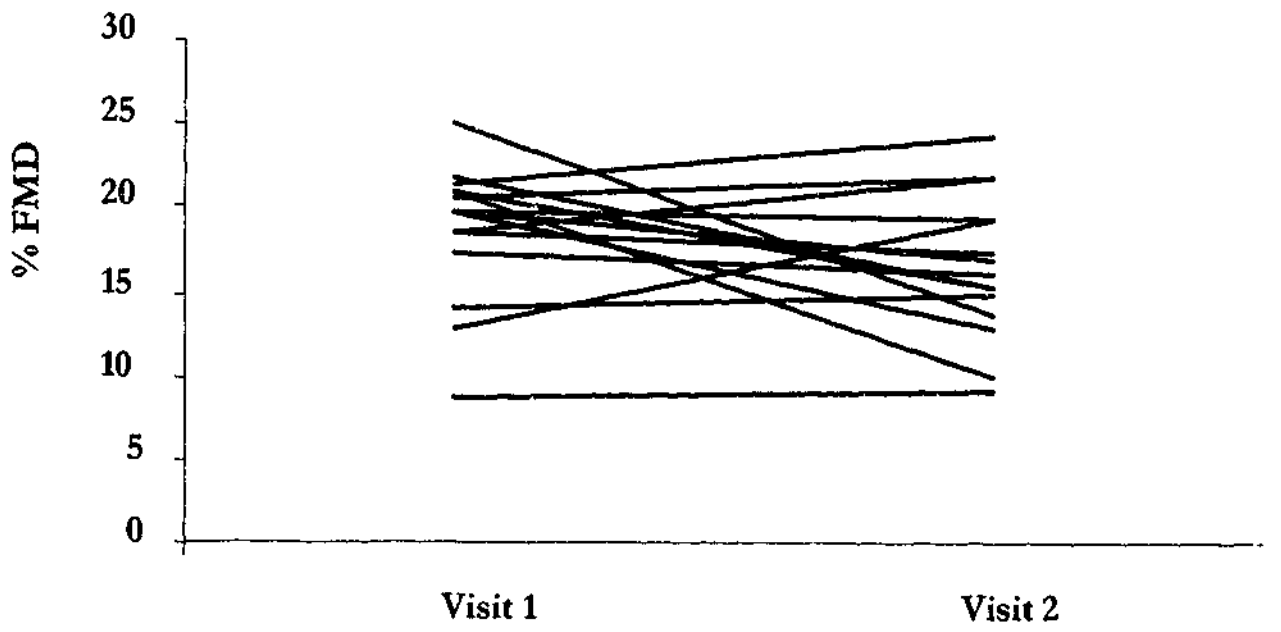
Data are presented as individual patient central pulse pressures.

* denotes a significant difference in mean central pulse pressure ± SEM, compared to baseline

A


*
25
20
15
10
5
0
% FMD
Visit 1
Visit 2

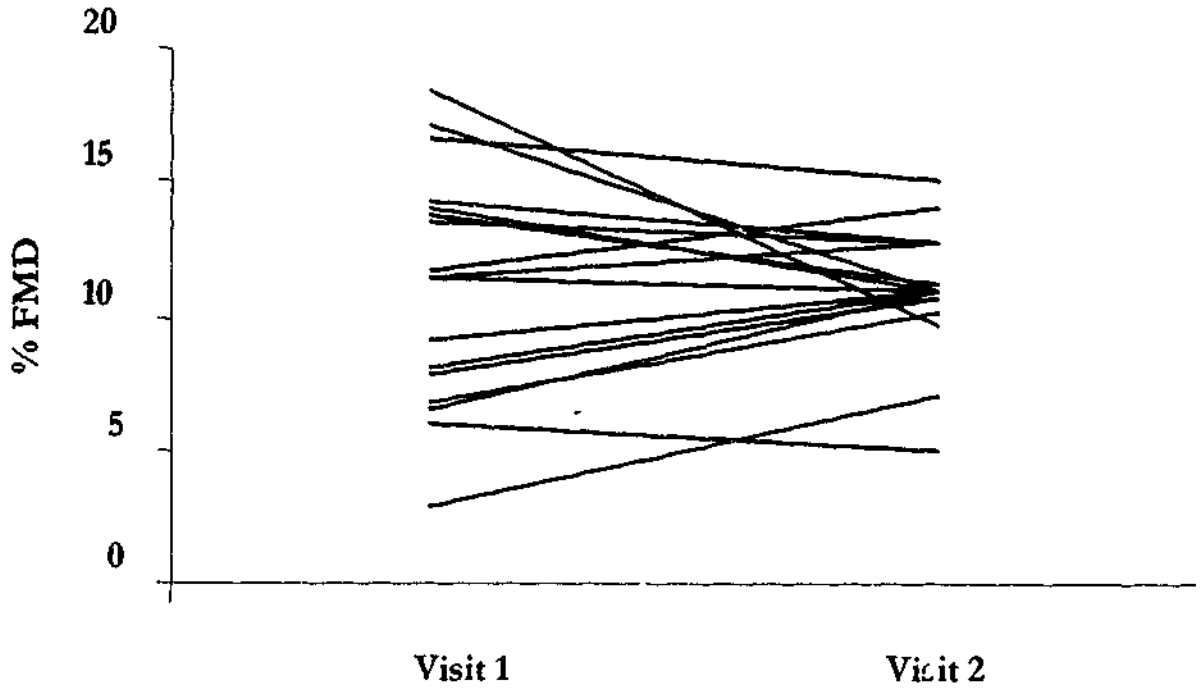
B


30
25
20
15
10
5
0
% FMD
Visit 1
Visit 2

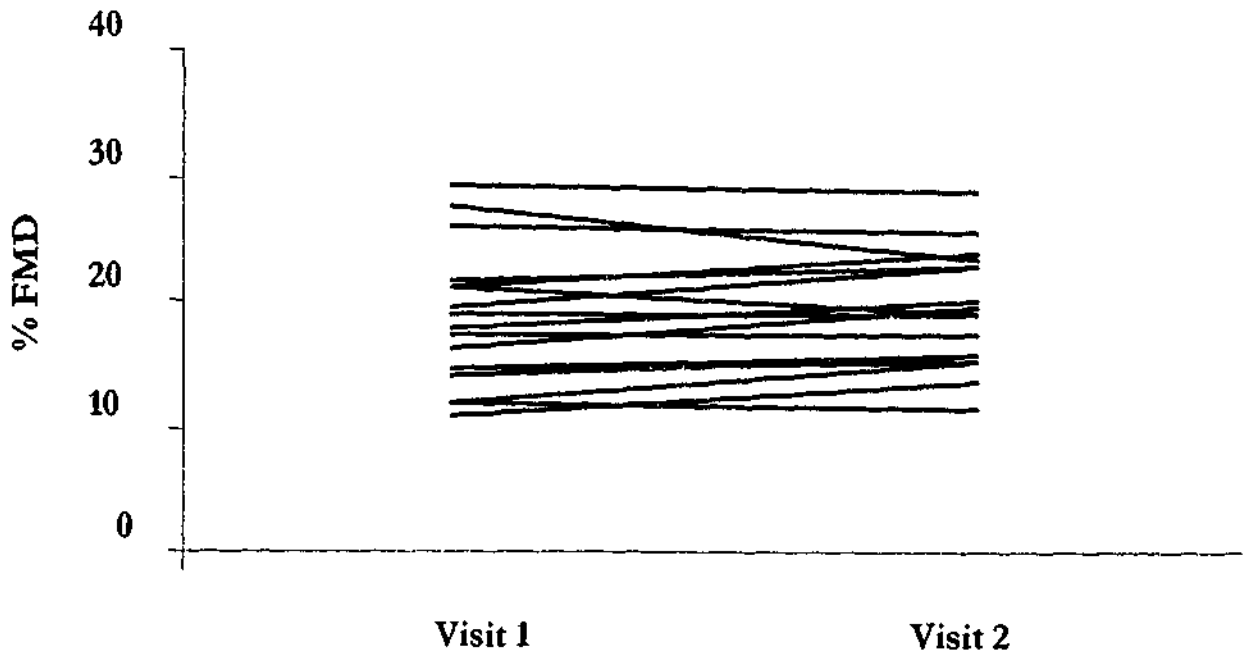
Appendix Figure 6 Effects of DHEA on %FMD in Healthy Postmenopausal Women
Mean %FMD of the brachial artery with reactive hyperemia is significantly increased following three months of DHEA administration **(A)** (8.4 ± 0.7 to 14.5 ± 1.1%, $p<0.05$). There were no changes in FMD following placebo treatment **(B)** (10.8 ± 1.1 to 10.9 ± 0.6%).

Data are presented as individual patient percentage FMD.
* denotes a significant difference in %FMD ± SEM be veen measurement time points.

A



B

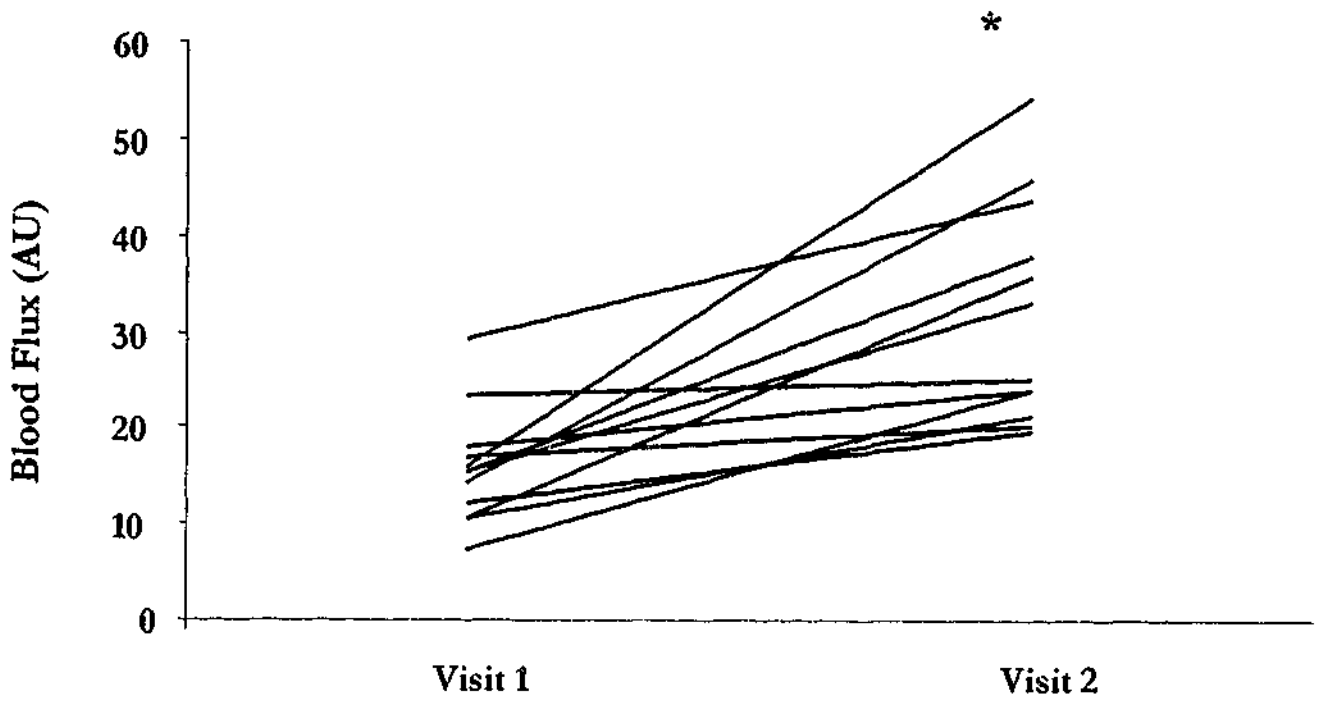


Appendix Figure 7 Effects of DHEA on %FMD following sublingual Glyceryl trinitrate Administration in Healthy Postmenopausal Women

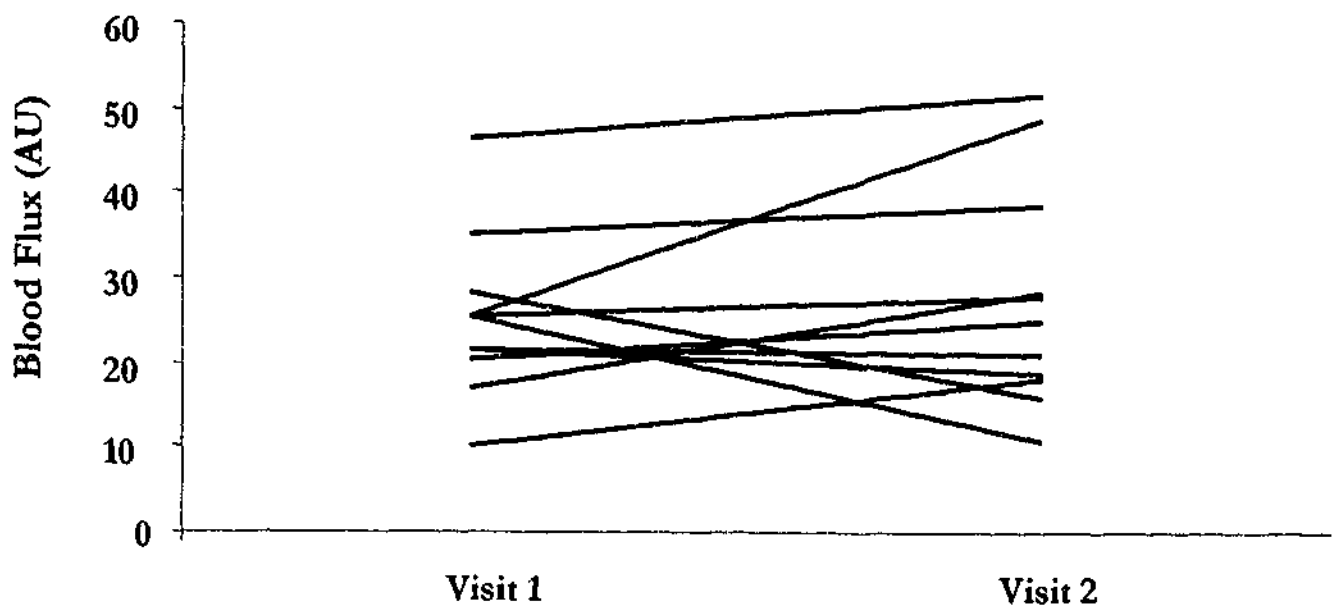
Sublingual glyceryl trinitrate (GTN)-induced endothelium-independent vasodilation was unaltered between measurement time points in both DHEA **(A)** and placebo **(B)** treatment groups (18.5 ± 1.1% compared to 16.4 ± 1.2% for DHEA and 18.4 ± 1.4% compared to 19.2 ± 1.3% for placebo treatment, p= 0.2 and 0.7 respectively).

Data are presented as individual patient percentage FMD ± SEM.

A


*
60
50
40
30
20
10
0
Blood Flux (AU)
Visit 1
Visit 2

B


60
50
40
30
20
10
0
Blood Flux (AU)
Visit 1
Visit 2

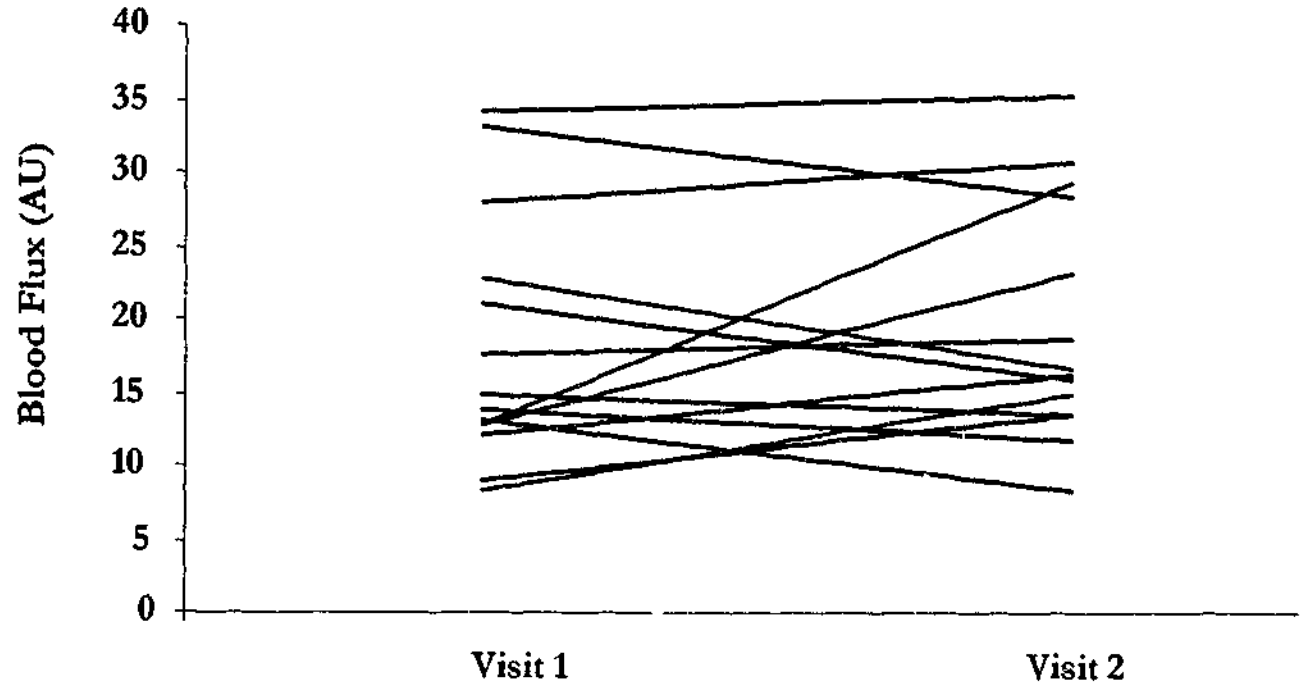
Appendix Figure 8 Effects of DHEA on Cutaneous Vascular Reactivity in Healthy Postmenopausal Women

Endothelium-dependent subcutaneous blood flow in response to iontophoresed ACh was significantly increased following DHEA administration compared with baseline values **(A)** (16 ± 2AU compared to 31 ± 3AU, $p<0.05$) . There were no differences observed in ACh-induced dilation between measurement time points in the placebo treated group **(B)** (18 ± 2AU compared to 20 ± 2AU).

Data are presented as mean arbitrary flux units (AU) for individual patients.

* denotes a significant difference in mean blood flux ± SEM between measurement time points.

A


Blood Flux (AU)
40
35
30
25
20
15
10
5
0
Visit 1
Visit 2

B

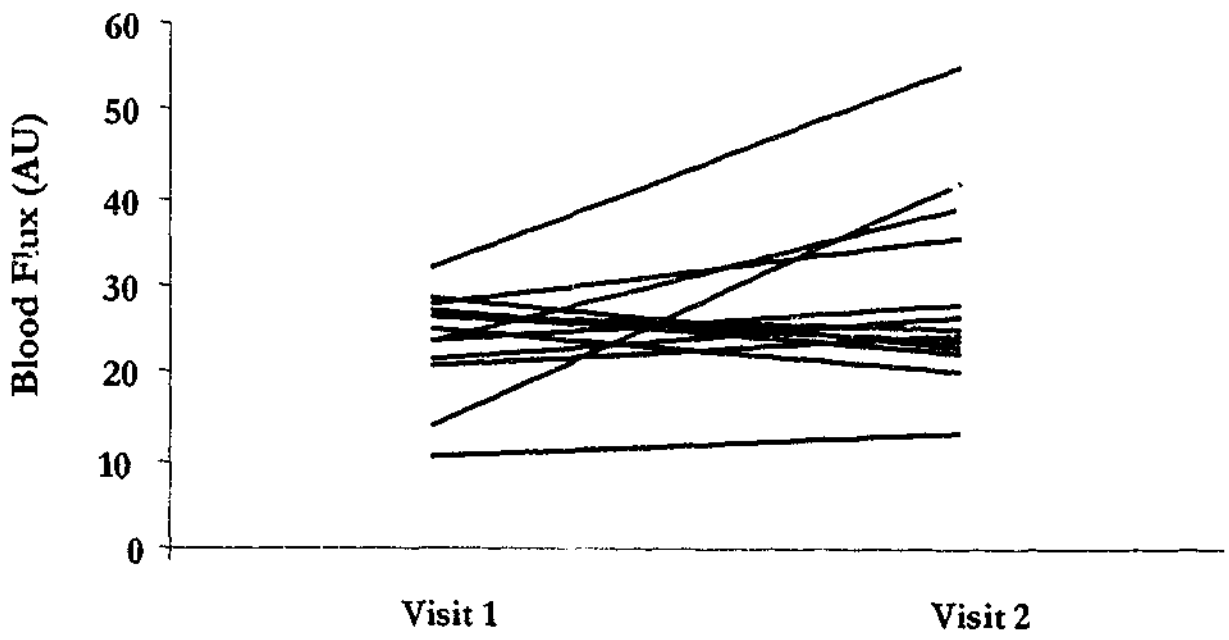
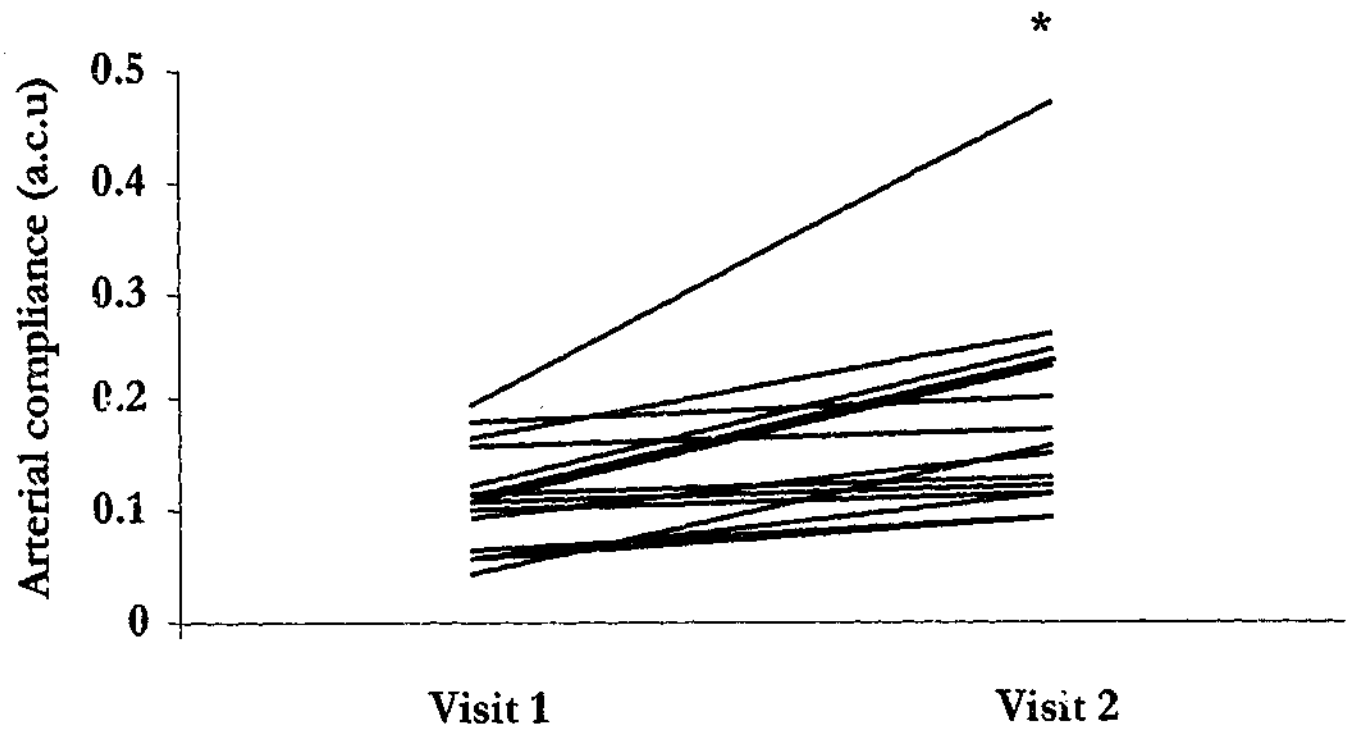

Blood Flux (AU)
60
50
40
30
20
10
0
Visit 1
Visit 2

Figure 9 Effects of DHEA on Cutaneous Vascular Reactivity in Healthy Postmenopausal Women

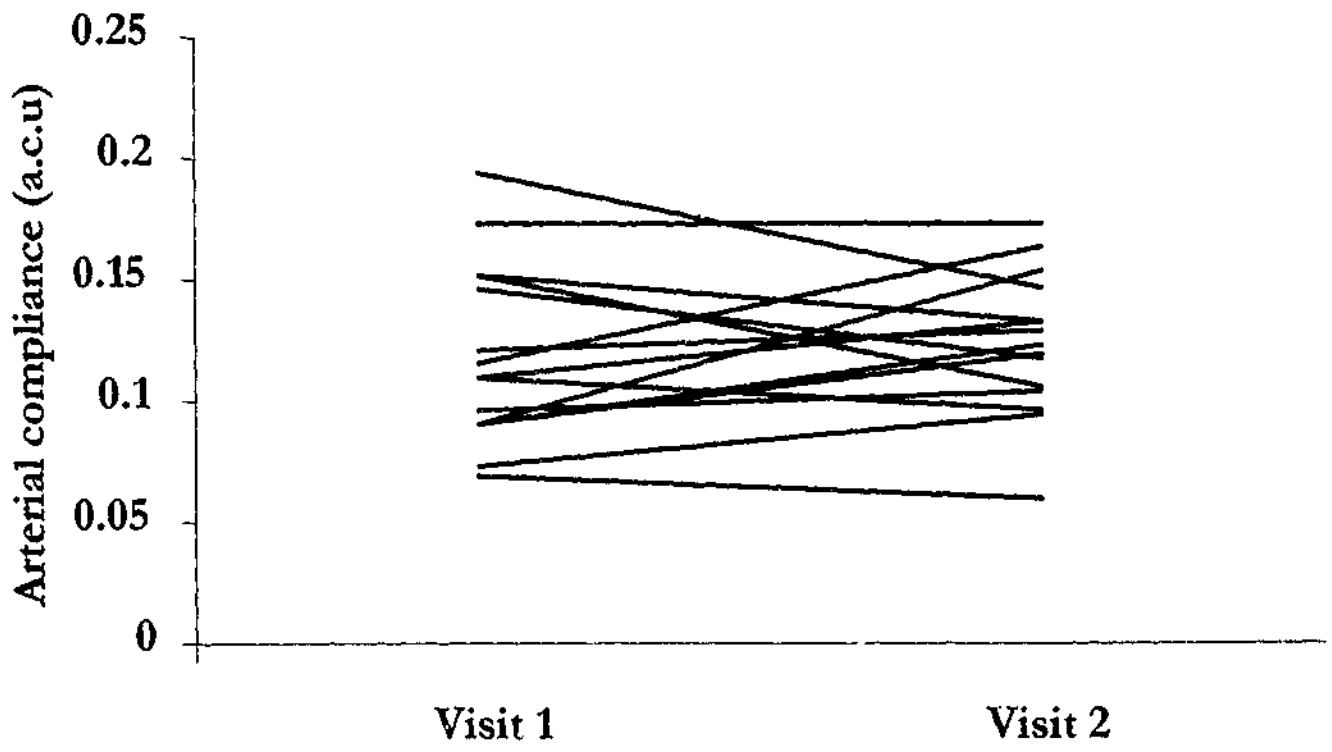
Endothelium-independent subcutaneous blood flux in response to SNP ionotophoresis was unaltered between the measurement time points in both DHEA and placebo treatment groups (25 ± 3AU compared to 28 ± 4AU for DHEA **(A)** and 24 ± 3AU compared to 28 ± 6AU for placebo treatment **(B)**, p= 0.67 and 0.2 respectively).

Data are presented as mean arbitrary flux units (AU) for individual patients.

A


*
Arterial compliance (a.c.u)
0.5
0.4
0.3
0.2
0.1
0
Visit 1
Visit 2

B


Arterial compliance (a.c.u)
0.25
0.2
0.15
0.1
0.05
0
Visit 1
Visit 2

Appendix Figure 10 Effects of DHEA on Systemic Arterial Compliance in Healthy Postmenopausal Women

SAC was significantly increased following DHEA administration compared with baseline values **(A)** (0.12 ± 0.01 compared to 0.18 ± 0.02a.c.u , $p<0.05$). There were no differences observed in SAC between measurement time points in the placebo treated group **(B)** (0.12 ± 0.01 compared to 0.12 ± 0.01).

Data are presented as arbitrary compliance units (a.c.u) for individual patients.

* denotes a significant difference in mean a.c.u ± SEM between measurement time points.