MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 4 May 2004.....
.....[redacted].....
Sec. Research Graduate School Committee

Addendum

Insert on page 118 after paragraph 2. In the original triglyceride study, chapter 2, animals were on a J129Sv x C57/black six background and at 6 months of aged presented with a severe hepatic steatosis phenotype. This led to significant changes in genes encoding ezymes involved in fatty acid synthesis and fatty acid uptake, in particular ACCα and SR-B1, when estrogen was removed. However, the animals that were examined for the estrogen replacement study, chapter 3, were also believed to be on the same background, however due to their less serve presentation of the hepatic steatosis phenotype and other common ARKO phenotypes they were later found to be prominently of the J129Sv background rather that the mixed background. In addition to this the animals from our colony were infected with helicobacter which caused the mice to become ill, and had some weight lose which also contributed to the reduction of the hepatic steatosis phenotype. It is believed that these changes in the background and the health of the animals led to the reductions in the severity of the hepatic steatosis in the animals discussed in chapter 3 compared with those in chapter 2. This results in a less dramatic changes in to ACCα and SR-B1 than first observed. Currently the mice are being breed on the C57black 6 background as it is kknown to be a better background of obese mice phenotypes.

Page 21, line 2, reference "Holak et al, 1988" should be replaced with "Kumar et al, 1970".

Page 23, section 1.322, line 10, "Singh & Poulos" should be replaced with "Kotti et al, 2000".

Page 50, reference inserted after reference 11 "Kotti TJ, Savolainen K, Helander HM, Yagi A, Novikov DK, Kalkkinen N, Conzelmann E, Hiltunen JK, Schmitz W 2000 In mouse alpha -methylacyl-CoA racemase, the same gene product is simultaneously located in mitochondria and peroxisomes. J Biol Chem. 2000 Jul 7;275(27):20887-95.

Page 34, paragraph 3, line 2, "Schoonjans et al, 2000" replace with "Horton et al, 2002"

Page 49, reference inserted after reference number 8 "Horton JD, Goldstein JL, and Brown MS 2002 SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver J. Clin. Invest. 2002 **109**:1125-1131.

Page 35, section 1.44, line 4 "Russell & Setchell, 1992" should be replaced with "Russell, 2003".

Page 57 reference inserted after reference number 3 "Russell DW 2003 The enzymes, regulation, and genetics of bile acid synthesis Annu Rev Biochem. 2003;72:137-74.

Page 3 Figure 1.1 double bonds in estrone should be between carbons 3 and 4 rathethan carbons 4 and 5

Page 9 line 1 a mutation in the ERα gene results in estrogen deficiency should be replaced with a mutation in the ERα gene results in impaired estrogen signalling.

Page 5, line 4, page 7 paragraph 2 lines 5, 6, 7, 8 "% homology" should be repaced with "amino acid similarity".

Page 22 paragraph 2 line 8 "long chain fatty acids" should proceed "LCFA".

Page 23 paragraph 2 line 7 "very long chain fatty acids" should proceed "VLCFA".

Pages 61, 90 and 106 under method section headed Mice, the mice were on a C57/black 6 background should be inserted.

Page 34, paragraph 2, line 10, "The COOH-terminal end regularly domain" should be deleted.

Errata

Pages 71 and 94 "p=0.000" should be replaced with "p<0.000".

Fig 3 chapter 3 NS should be proceeded with "non significant".

Appendix figure 5 line 1 "gonad", should be replaced with "gonadal", line 4 "elemen" should be replaced with "element" and line 5 "presente" should be replaced with "presented".

Page 4, line 6 "exon" should be replaced with "exons".

Page 6, section 1.221, line 3 "in" should be deleted.
Page 26, line 3 "signifcant" should be replaced with "significant".
Page 26, line 15 "lysosmes" should be replaced with "lysosomes".
Page 29, line 15 "isoprenes" should be replaced with "isoprene".
Page 32, line 1, "mediate" should be replaced with "mediates".
Page 32 line 3, "pinching off form" should be replaced with "pinching off to form".
Page 67, paragraph 2, line 8 "cholesterol high diet" should be replaced with "high cholesterol diet".
Page 68, paragraph 3, line 2 "surcose" should be replaced with "sucrose".
Page 73, paragraph 2, line 5 "and" should be deleted.
Page 120, paragraph 2, line 4 "with have large" should be replaced with "have large".

The Liver Phenotype of the Aromatase Knockout (ArKO) mouse

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Thesis submitted for the degree of Doctor of Philosophy

Prince Henry's Institute of Medical Research

and

Department of Biochemistry and Molecular Biology,

Monash University

2003

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General Declaration

In accordance with Monash University Doctorate Regulation 17 / Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original paper published in peer reviewed journals and 3 publications currently submitted, 1 accepted with minor revisions, 1 in revision and supporting data in an appendix is in review. The core theme of the thesis is the regulation of lipids by estrogen. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Prince Henry's Institute of Medical Research and through the Department Biochemistry and Molecular Biology under the supervision of Professor Evan Simpson and Dr. Margaret Jones.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters two, three and four my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
two	Estrogen deficiency leads to a sexually dimorphic phenotype of hepatic steatosis in male mice.	In Revision, Endocrinology	100% experimental contribution 89% overall
three	Estrogen replacement reverses	Accepted to	97% experimental

	the hepatic steatosis phenotype in the male aromatase knockout (ArKO) mouse	Endocrinology pending minor revisions	contribution 90% overall
Four	The aromatase knockout mouse presents with a sexually dimorphic disruption to cholesterol homeostasis	Published, Endocrinology, 144(9):3895-3903	100% experimental 89% overall
Appendix one	Cholesterol feeding prevents adipocyte hypertrophy in the obese female aromatase knockout (ArKO) mouse	In review, Endocrinology	3% experimental

Signed: [redacted] .....

Date: 10/11/2002

Acknowledgments

There are many people to thank for their help over the past three and half years. Firstly, I would like to thank Professor Evan Simpson for allowing me to study in your group and under your supervision. Hope you have eggs for breakfast every morning now, I do! Also for allowing me two overseas trips, firstly US Endo 2002, it was a truly inspiring trip. Secondly, for my trip to Europe in 2003, which gave me the opportunity to visit laboratories and to find my postdoctoral position with Professor Paul Stewart.

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word! And the other members of the awesome foursome, Aggie, Natalie and Christine, thanks for making me spin, it has kept me somewhat sane during my write up, so next year will it be the awesome threesome?

To my all friends, Bek, Amber, Jo and Em, thanks guys for your support and patience, particularly of these last few months and the next whamarama will have to be in its true home of England. And the whole Seymour gang for always being up for a beer! Also to Kaz, thanks for your friendship and excellent suggestions for a quick and easy write up! Also my Ararat friends, Melissa, Nicole and Mandy, sorry for boring you all with my science talk! I would also like to thank all of those people who I have shared housed with over the past few years, I think it's been ten. But in particular I would like to thank Sue, Gen, Chelsea and Christine, your support and friendships have been invaluable, also leaving the couch free for me and keeping the noise down during nap time!

Last but certainly not least, is my family, thanks to all of you. Mum and Dad for all your support, wisdom and love and I know when you signed up for parenthood you didn't think you would a child that would never leave university. Also for trying to keep my car going, we would have made it if not for the four-wheel drive! Also to Melissa, Simon, Brent and Aaron, thanks guys for your support and letting me off the hook in present donations and shouting me at the pub when I have no money.

Thanks everybody and I hope to see you at 'the oval' in Birmingham for the 2005 Ashes tour!

List of Publications and Presentations

Refereed Journal Articles

Hewitt KN, Boon WC, Murata Y, Jones MEE & Simpson ER The Aromatase Knockout (ArKO) Mouse Presents with a Sexually Dimorphic Disruption to Cholesterol Homeostasis 2003 Endocrinology 144(9):3895-3905.

Hewitt KN, Boon WC, Murata Y, Jones MEE & Simpson ER Estrogen Deficiency Leads to a Sexually Dimorphic Phenotype of Hepatic Steatosis in Male Mice 2003 Submitted to Endocrinology, In Revision.

Hewitt KN, Pratis K, Jones MEE & Simpson ER Estrogen Replacement Reverses the Hepatic Steatosis Phenotype in the Male Aromatase Knockout (ArKO) Mouse 2003 Accepted to Endocrinology pending minor revisions.

Jones MEE, Thornburn AW, Britt KL, **Hewitt KN**, Wreford NG, Proietto J, Oz O, Leury B, Robertson KM & Simpson ER Aromatase deficient (ArKO) mice have a phenotype of increased adiposity 2000 Proc. Natl. Acad. Sci. USA 97: 12735-12740.

Misso ML, **Hewitt KN**, Boon WC, Murata Y, Jones MEE & Simpson ER Cholesterol feeding prevents adipocyte hypertrophy in obese female aromatase knockout (ArKO) mouse 2003 Submitted to Endocrinology In Revision.

Invited Conference Proceedings

Jones MEE, Thornburn AW, Britt KL, **Hewitt KN**, Misso ML, Wreford NG, Proietto J, Oz O, Leury B, Robertson KM, Yao S & Simpson ER Aromatase deficient (ArKO) mice have a phenotype of increased adiposity. (2001) The Journal of Steroid Biochemistry and Molecular Biology: 79: 3-9.

Abstracts and Presentations for conferences

Jones MEE **Hewitt KN**, Britt KL, Thornburn AW, Robertson KM, Proietto J & Simpson ER (February, 2000). Oestrogen deficiency in the aromatase knockout (ArKO) mouse produces an obese phenotype. 12 th Lorne Cancer Conference. Lorne, Victoria, Australia.

Jones MEE, Thornburn AW, **Hewitt KN**, Britt KL, Oz OK, Leury BJ, Murata Y, Misso ML, Boon WC & Simpson ER. (November, 2000). The obese phenotype of the ArKO mouse. Aromatase 2000 and 3 rd generation. Port Douglas, Queensland, Australia.

Hewitt KN, Boon WC, Murata Y, Simpson ER & Jones MEE (September 2001) Oestrogen deficiency in the aromatase knockout (ArKO) mouse leads to lipid accumulation in the liver. ESA, Gold Coast, QLD, Australia.

Hewitt KN, Boon WC, Murata Y, Simpson ER & Jones MEE Oestrogen deficiency in the aromatase knockout (ArKO) mouse leads to lipid accumulation in the liver. Submitted for 84th Annual U.S. Endocrine Society San Francisco, 2002.

Misso ML, **Hewitt KN**, Boon WC, Simpson ER, & Jones MEE. Cholesterol reverses adipocyte hypertrophy in the obese aromatase knockout (ArKO) mouse. Submitted for 84th Annual U.S. Endocrine Society San Francisco, 2002.

Jones MEE, **Hewitt KN**, Misso ML, Boon WC, Murata Y, Chung J, Kawaguchi S & Simpson ER. Aromatase-deficient (ArKO) mice accumulate excess adipose tissue. Submitted 9th International Congress on Obesity, August 24-29, 2002, Sao Paulo, Brazil.

Hewitt KN, Boon WC, Murata Y, Simpson ER & Jones MEE (September, 2002). Cholesterol feeding rescues the fatty liver phenotype of the aromatase knockout (ArKO) mouse. ESA, Adelaide, S.A., Australia.

Hewitt KN, Boon WC, Murata Y, Jones MEE & Simpson ER The aromatase knockout (ArKO) mouse presents with a sexually dimorphic liver phenotype. 6th European Congress of Endocrinology Lyon, France, April 2003.

Abbreviations

ACAT2	acylCoA:cholesterol acyltransferase 2
ACCα	acetyl-CoA carboxylase α
ACCβ	acetyl-CoA carboxylase β
ACP	acyl carrier protein
ACD	acyl-CoA dehydrogenase
ACO	acyl-CoA oxidase
ADRP	adipocyte differentiated regulatory protein
ANOVA	analysis of variance
AOX	peroxisomal acyl-CoA oxidase
Apo	apoprotein
Arc	arcuate nucleus
ArKO	Aromatase knockout mouse
ATP	adenosine triphospahte
BAT	brown adipose tissue
cAMP	cyclic adenosine monophosphate
CTP1	carnitine palmitoyl transferase I
CTP2	carnitine palmitoyl transferase II
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
DNL	*de novo* lipogenesis
ECH	enoyl-CoA hydratase
E_2	17β-estradiol

ER	estrogen receptor
αERKO	estrogen receptor α knockout mouse
βERKO	estrogen receptor β knockout mouse
αβERKO	estrogen receptor αβ knockout mouse
FAS	fatty acid synthase
FATP	fatty acid transporter protein
FA	fatty acid
FFA	free fatty acid
FSH	follicle stimulating hormone
FXR	farnesoid X receptor
g	gram (s)
GH	growth hormone
HBR	heme binding region
HDL	high-density lipoprotein
HMG-CoA	β-hydroxy-βmethylglutayl-CoA
HRT	hormone replacement therapy
HSL	hormone sensitive lipase
H_2O	water
IGF-1	insulin-like growth factor type 1
IGF-2	insulin-like growth factor type 2
IR	insulin resistance
kg	kilogram (s)
LCFA	long chain fatty acid
LDL	low-density lipoprotein

LDLR	low-density lipoprotein receptor
LH	luteinising hormone
LPL	lipoprotein lipase
LRH-1	liver receptor homolog-1
LXR	liver X receptor
LXR-RE	liver X receptor response element
MCFA	medium chain fatty acid
MCAD	medium-chain acyl-CoA dehydrogenase
ME	medial eminence
mg	milligram (s)
MOP	medial preoptic area
MTTP	microsomal triglyceride transfer protein
NADPH	reduced nicotinamide adenine dinucleotide phosphate
Neo	neomycin resistant
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	9-cis retinoic acid receptor
SCAP	SREBP cleavage activating protein
SCFA	short chain fatty acid
SEM	standard error mean
SHP	short heterodimer partner
S1P	site 1 protease

S2P	site 2 protease
SR-B1	scavenger receptor class b type 1
SRE	sterol response element
SREBP	sterol regulatory binding protein
TGFα	transforming growth factor α
TK	thymidine kinase
μg	microgram (s)
UTR	untranslated region
VLACS	very long chain fatty acyl-CoA synthetase
VLDL	very low-density lipoprotein
v/v	volume per volume
WT	wildtype

Abstract

Cardiovascular disease is the number one cause of death in the western world, therefore understanding the mechanisms which contribute to this disease are vital to its treatment. Serum low-density lipoproteins (LDL) positively correlate with cardiovascular disease and are elevated in men and postmenopausal women compared with premenopausal women, implying that estrogen may play a role in their regulation. Additionally, triglyceride levels are also elevated in a state of low estrogen, so a role for estrogen in regulating lipid homeostasis begins to become apparent.

To further investigate this possible role of estrogen, I utilised the aromatase knockout (ArKO) mouse, an estrogen deficient model. Previously, estrogen deficient males and females have been shown to be obese with elevated levels of serum cholesterol and high-density lipoprotein (HDL) and at one year of age males had elevated serum triglycerides levels. Additionally, in older mice a fatty liver was observed. Therefore the aims of this thesis were to study the hepatic phenotypes of the estrogen deficient ArKO mouse to gain an understanding of the role of estrogen in lipid homeostasis and to use high cholesterol diets to further challenge cholesterol homeostasis.

The work in this thesis shows that the absence of estrogen led to sexually dimorphic phenotypes of lipid homeostasis in six month old mice. The absence of estrogen lead to a more dramatic phenotype in male mice when examining triglyceride homeostasis. Estrogen deficient males present with hepatic steatosis, due to elevated hepatic triglyceride levels. This may be due, in part, to elevations in the expression of enzymes involved in *de novo* lipogenesis, specifically fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACCα). Six weeks of estradiol treatment beginning at 18 weeks of age, was able to reverse the hepatic steatosis phenotype in the ArKO males by reducing hepatic triglyceride levels. This also

reduced FAS expression, indicating reduced fatty acid synthesis. Estrogen deficient females however only presented with elevations in serum triglycerides at this age and no alteration to hepatic triglycerides.

Both genders had disrupted cholesterol homeostasis, however it did manifest with different presentations. Estrogen deficient males presented with elevated hepatic cholesterol levels and no changes to serum cholesterol levels. Conversely, females presented with decreases in hepatic cholesterol levels and elevated serum cholesterol and HDL levels. Gene expression studies were used to examine key enzymes in cholesterol biosynthesis and excretion and in males there were no changes, possibly indicating that estrogen regulates these proteins posttranscriptionally. Estrogen deficiency in females led to a decrease in *Cyp7a* and ACAT2 expression, no other changes were seen.

Surprisingly, cholesterol feeding reversed the previously described obese phenotypes in both genders. Additionally, it reversed the hepatic steatosis in the males, by reducing hepatic triglyceride levels. However, despite the gender-specific disruptions to cholesterol homeostasis, the addition of cholesterol to the diet led to similar effects within genders regardless of estrogen status.

In conclusion, the work described here shows that estrogen has an important role in lipid homeostasis in both males and females, however its actions differ between the sexes.

Chapter One: Introduction and Literature Review

1.1 Introduction

Cardiovascular disease is the number one cause of death in the western world. Epidemiological studies show that high serum low-density lipoprotein (LDL) cholesterol levels positively correlate with cardiovascular disease (Castelli *et al.* 1977), conversely elevated high density lipoprotein (HDL) levels are negatively associated (Gordon *et al.* 1977). Additionally, there is increasing evidence to suggest that evaluated serum triglyceride levels also play an important role in predicting cardiovascular health, hence understanding factors that regulate cholesterol and triglyceride homeostasis are important in the prevention and treatment of these diseases.

There are many factors that regulate plasma lipid levels, such as production, uptake and excretion. Epidemiological studies have revealed a role for estrogen in regulating these metabolic activities. After menopause, the ovaries no longer produce estrogen thereby severely reducing circulating estrogen levels, to a level similar to adult men (Simpson *et al.* 2000). In the absence of estrogen there is a shift in body fat distribution from the hip region to the abdominal region (Tchernof *et al.* 1998; Tchernof & Poehlman 2000; Simkin-Silverman & Wing 2000; Sites *et al.* 2001; Garaulet *et al.* 2002), without necessarily a change in body weight and thus postmenopausal women present with fat distribution similar to men, implying estrogen plays a key role in body fat distribution. An increase in abdominal fat has been associated with insulin resistance and the 'metabolic syndrome' (Meigs 2002). This increased abdominal rather than in the peripheral fat is also associated with a higher incidence of cardiovascular disease (Kannel *et al.* 1991). In addition, postmenopausal women have altered circulating cholesterol levels, specifically increased LDL and a decrease in HDL

levels (Heiss *et al.* 1980; Kuller *et al.* 1990). Serum triglyceride levels are also increased in postmenopausal woman compared with premenopausal women. Interestingly, hormone replacement therapy (HRT) has been shown to reduce LDL levels and increase HDL levels, again implying a positive effect of estrogen on cholesterol levels (Erberich *et al.* 2002). Men have lower circulating levels of estrogen compared with premenopausal women and they have comparatively higher serum LDL and triglyceride levels, a features as also observed in postmenopausal women (Carr 2003). This provides further evidence that estrogen may positively regulate lipid homeostasis.

Tamoxifen is an estrogen receptor antagonist, which is used in the treatment of breast cancer. Some patients treated with tamoxifen present with hepatic steatosis, which is generally due to an accumulation of triglycerides. Subsequent withdrawal of treatment can reverse this side effect (Murata *et al.* 2000; Nemoto *et al.* 2002; Coskun *et al.* 2002; Ogawa *et al.* 2003; Nishino *et al.* 2003; Murata *et al.* 2003). Collectively this evidence reveals a role for estrogen in the regulation of lipid homeostasis.

1.2 Estrogen biosynthesis and signalling

Estrogen is synthesised by the conversion of testosterone to 17β-estradiol and androstenedione to estrone. Both of these reactions are catalysed by the enzyme aromatase P450 (Figure 1.1). 17β-estradiol is the more biologically active estrogen, whereas estrone is a weaker compound. Steroid hormones can act in a paracrine, autocrine and endocrine fashion and estrogen exerts its effects through signalling through specific receptors, known as estrogen receptor α (ERα) and estrogen receptor β (ERβ).

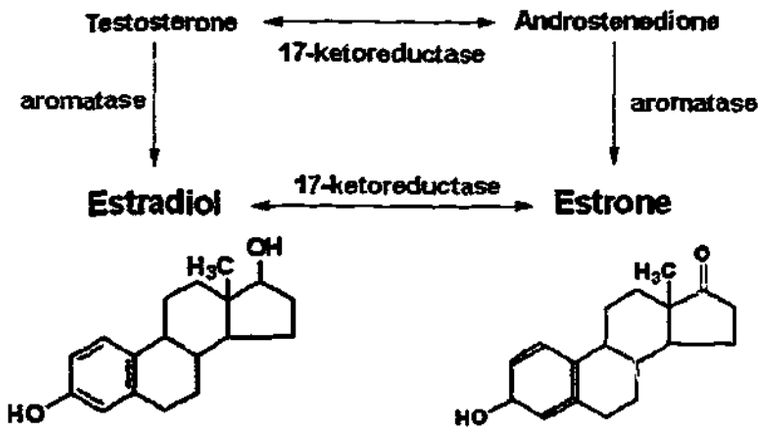


Figure 1.1: The conversion of testosterone to estradiol and androstenedione to estrone. Both reactions are catalysed by the enzyme aromatase P450 (diagram reproduced from http://www.med.unibs.it/~marchesi/sterhorm.html)

1.21 Aromatase

1.211 *CYP19* gene structure

Human aromatase P450 is encoded by the *CYP19* gene which is located on chromosome 15q21.2 (Chen *et al.* 1988). It was cloned and characterised over a decade ago and is a member of the P450 superfamily of genes (Means *et al.* 1989; Harada *et al.* 1990; Toda *et al.* 1990). *CYP19* has a coding region of ten exons where the translation initation codon is localised in exon II (Figure 1.2). The exons II to X are distributed across approximately 34.3 kb of the genome (Sebastian & Bulun 2001). The untranslated 5' terminus contains at least five untranslated exons, I.1, I.3, I.4, If and IIa which are driven by tissue specific promoters therefore are expressed in a tissue specific fashion (Means *et al.* 1991; Kilgore *et al.* 1992; Jenkins *et al.* 1993; Toda & Shizuta 1993). This untranslated region is distributed across approximately 96.5 kb (Sebastian & Bulun 2001). Promoter I.1 is located at least 90kb upstream from the translation start site and drives expression in the placenta (Means *et al.* 1991; Mahendroo *et al.* 1991; Kamat *et al.* 1999). Conversely, expression in the ovary is driven by promoter II which is located directly upstream of the start codon (Jenkins *et al.* 1993). Promoter I.4 is the main promoter driving aromatase expression in adipose tissue and

like promoter I.1 it is also distal to the start site and is about 20 kb downstream from promoter I.1 (Mahendroo *et al.* 1991). Promoter I.3, which is located about 0.25kb upstream of the start site has also been shown to drive aromatase expression in adipose tissue (Kamat *et al.* 2002). The brain specific promoter, If is located approximately 40 kb upstream of the start codon (Kamat *et al.* 2002). Transcripts from these tissue specific promoters always produce the same protein as splicing of these untranslated exon occurs at a common 3'-splice junction, which is located upstream of the start of translation.

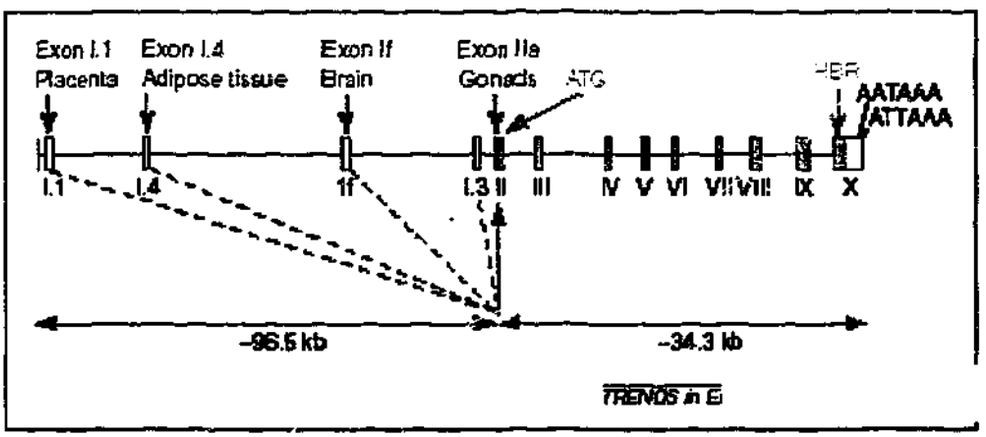


Figure 1.2: Schematic representation of the human *CYP19* gene. Exons II-X are shown in purple, and together with the corresponding introns comprise a region of 34.3 kb. Exon X contains the heme binding region (HBR) and two polyadenylation signals present in the 3'-untranslated region (UTR) (yellow). The 5' UTR (yellow) contains the five untranslated exons, I.1, 1.4, If, I.3 and IIa, this region is approximately 96.5 kb. Alternate splicing occurs at the common start site just upstream of the ATG codon at exon II. Reproduced from (Kamat *et al.* 2002).

The different promoters are regulated by different mechanisms, for example promoter II, which drives aromatase expression in ovaries, is stimulated by follicle-stimulating hormone (FSH) which is mediated by cyclic adenosine monophophate (cAMP) (Simpson *et al.* 1997b). Promoter I.1 which drives aromatase expression in the placenta is regulated by retinoids (Sun *et al.* 1998). Class I cytokines are known to drive aromatase expression in adipose tissue using promoter I.4 (Mahendroo *et al.* 1993; Zhao *et al.* 1995).

The cDNA encoding the mouse aromatase was cloned in 1991 (Terashima *et al.* 1991) and revealed a 1.5kb open reading frame with a 46bp 5'untranslated region and a 839bp 3'untranslated region, containing a poly (A) tail. This encodes a protein comprised of 503 amino acids, which shares 91 and 81% homology with the rat and human amino acid sequences, respectively. The mouse aromatase cDNA also contains the heme-binding domain which is highly conserved across many species and is responsible for the catalytic activity of the enzyme (Simpson *et al.* 1997b). In the mouse, aromatase transcripts were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in the hypothalamus, ovary, testis and gonadal fat pad. The transcript was not detectable in the pituitary, skin, lung, liver or heart (Boon *et al.* 1999).

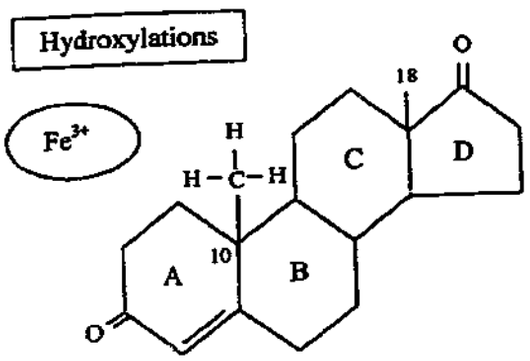
1.212 Estrogen Biosynthesis

As mentioned earlier, estrogen biosynthesis is catalysed by the enzyme aromatase P450. This enzyme binds to C_{19} steroids and catalyses a series of reactions, comprising two hydroxylation steps followed by an aromatisation step, during which the phenolic A ring characteristic of estrogens, is formed (Figure 1.3 reproduced from Robertson 2001). A flavoprotein, niocotinamide adenine dinuleotide phosphate (reduced) (NADPH)-cytochrome P450 reductase, is associated with aromatase (Simmons *et al.* 1985), regulating the transfer of reducing equivalents from NADPH to the cytochrome P450 (Simpson *et al.* 1997b). The reaction takes place in the endoplasmic reticulum of cells, where three moles of oxygen and three moles of NADPH are used for every mole of C_{19} steroid metabolised (Thompson, Jr. & Siiteri 1974). There are two hydroxylation reactions occurring at the C_{19} methyl group. The first reaction leads to the formation of 19-hydroxy-androstenedione and the second hydroxylation results in the formation of 19-oxo androstenedione. The third reaction is a peroxidative attack, where the C-19-oxo group removes a proton from the C1 position,

Figure 1.3 Estrogen Biosynthesis

The final step in the biosynthesis of estrogen is the conversion of androgens, which is catalysed by aromatase P450. There are two hydroxylation reactions. The first step to estrogens leads to the formation of 19-hydroxy-androstenedione and the second hydroxylation results in the formation of 19-oxo androstenedione. The aromatisation step leads to the deformylation of the C19 and aromatisation of the A ring, leading to the formation of estrogen. (Reproduced from Robertson, 2001).

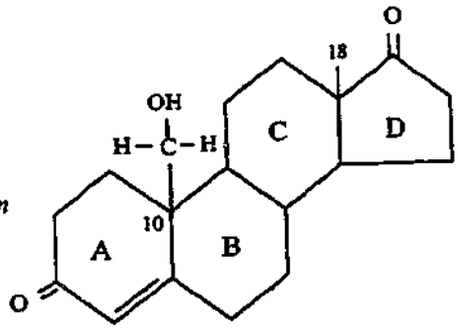
Hydroxylations



Androgen substrate (C19)

NADPH
O_2

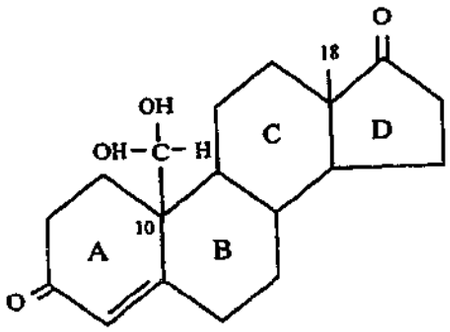
1st 19- hydroxylation



19-hydroxy-androstenedione

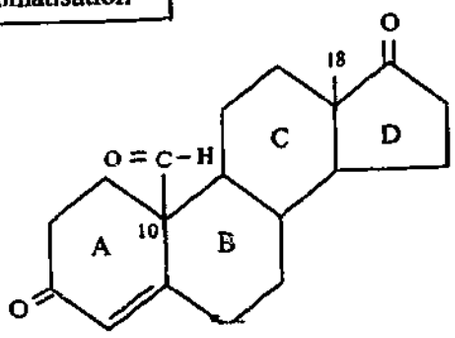
NADPH
O_2

2nd 19- hydroxylation

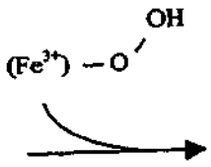


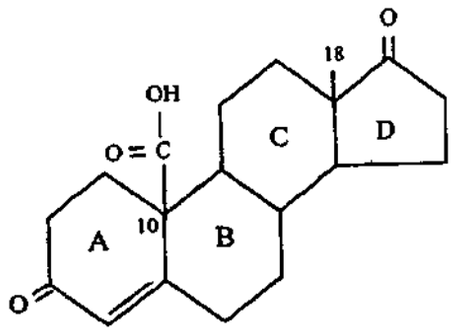
H_2O

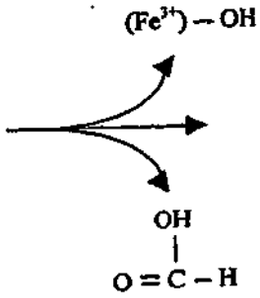
Aromatisation



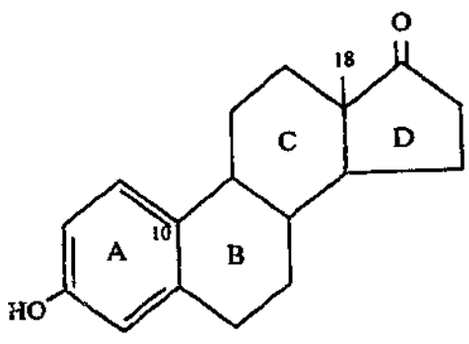
19-oxo androstenedione







(formic acid)



estrogen (C18)

leading to the deformylation of the C_{19} and the aromatisation of the A ring. Thus, estradiol is synthesised from testosterone while estrone is derived from androstenedione (Simpson *et al.* 1997b).

1.22 Estrogen receptors

Like all steroid hormones, estrogen exerts its effects through its specific receptors, ERα and ERβ. The human ER was cloned in 1986 from the human breast cancer cell line MCF-7 (Green *et al.* 1986) and the rodent ER was identified from the rat uterus in 1966 (Toft & Gorski 1966). In the mid nineties a second ER was identified in the prostate and ovary of the rat (Kuiper *et al.* 1996), in peripheral blood leukoctyes from humans (Mosselman *et al.* 1996) and from mouse ovaries (Tremblay *et al.* 1997). It was named ERβ, thereby renaming the first, ERα. Tissue distribution of the ERs in mouse revealed that transcripts of ERα have a wide distribution, including the central nervous system, breast, liver, bone, cardiovascular system, gastrointestinal tract and the urogenital tract (Gustafsson 1999), whereas ERβ is found mainly in the ovary, hypothalamus, lung and in the male reproductive tract (Couse & Korach 1999). Within cells ERα has been localised to the nucleus (Greene *et al.* 1980; Greene *et al.* 1984).

1.221 Estrogen Receptor Structure and Mechanisms of Action

The two ERs are not isoforms of each other, the genes are located on separate chromosomes and encode distinct proteins. Transcript from the mouse ERα result in a gene of nine exons, which is approximately 6.3kb, encoding a protein of 599 amino acids with a in molecular weight of approximately 66kDa (White *et al.* 1987). The ERβ protein is composed of 485

amino acids and has an estimated molecular weight of 54kDa (Kuiper *et al.* 1996; Tremblay *et al.* 1997; Pettersson *et al.* 1997).

Like other members of the superfamily of steroid/thyroid hormone nuclear receptors the ERα and ERβ proteins have six functional domains, labelled A-F with differing degrees of homology between the domains (Figure 1.4, reproduced from Gustafsson 1999). The N-terminal domain (NHD) consists of the A/B region, which contains the activation function 1 (AF1) domain, it has only 17% homology between ERα and ERβ. The DNA binding domain (DBD), C region, has the highest homology of between ERα and ERβ 97%. The hinge region, D domain, has little homology (30%) and contains nuclear localisation signals. The ligand binding domain (LBD), E region, has 59% homology. The C-terminus has the E/F region that contains the activation function (AF2) domain and both of these regions are involved in transactivation (White & Parker 1998).

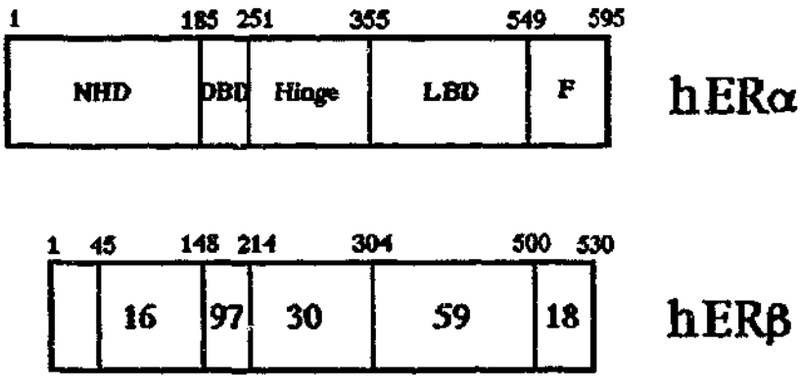


Figure 1.4: Structure of human ERs. The separate domains are identified in the ERα diagram; NHD refers to the N-terminal domain, DBD refers to DNA binding domain, is responsible for binding at estrogen response elements (ERE) on the chromosome. LBD refers to ligand binding domain, The numbers (%) in the ERβ diagram refer to sequence identity (Gustafsson 1999).

In the absence of estrogen the estrogen receptor is a monomer and is associated with heat shock proteins and other proteins (Smith 1993; Smith & Toft 1993). Once estrogen diffuses into the cell and binds to the ligand binding domain of the receptor this complex binds to the DNA, causing a conformational change in the estrogen receptor, leading to the dissociation of

the bound proteins (Smith 1993). The ER can then bind to the estrogen response element (ERE) in the DNA, which consists of two inverted repeats of A/GGGTCA separated by three nucleotides (White & Parker 1998). The complex can then interact with basal transcription factors and co-regulator proteins, which then lead to the transcription of target genes (Tsai & O'Malley 1994; Weigel & Zhang 1998; White & Parker 1998). Both the ERs are able to act as homo and heterodimers (Pettersson *et al.* 1997; Luconi *et al.* 2002) (Figure 1.5).

1.222 Nongenomic actions of estrogen

Estrogen actions take effect over a time frame ranging from seconds to hours. This difference led to the hypothesis of nongenomic actions of estrogen (Luconi *et al.* 2002), in addition to the already characterised classical genomic pathway of signalling through ERα and ERβ. The nongenomic actions have more rapid effects and they appear to be initiated at the plasma membrane level, but the mechanisms are still controversial. One hypothesis was established in MCF-7 cells, whereby estradiol was reported to stimulate proliferation in mammary cancer through the direct activation of Src at the plasma membrane. Membrane ER is able to form a complex with Src in the inner plasma membrane, leading to the activation of Src kinase, which through Ras/ERK, stimulates ERK, leading to cell proliferation (Luconi *et al.* 2002). Another mechanism has been shown where G-protein coupled membrane ER has also been characterised in murine macrophages and stimulation may be related to an increased calcium flux (Benten *et al.* 2001). Alternatively, estrogen may mediate effects through activation of associated endothelial nitric oxide synthase (eNOS). A subpopulation of ERα has been localised to the plasma membrane caveolae in endothelial cells. Plasma membranes from COS-7 cells expressing eNOS and ERα have displayed ER-mediated eNOS stimulation (Chambliss *et al.* 2000).

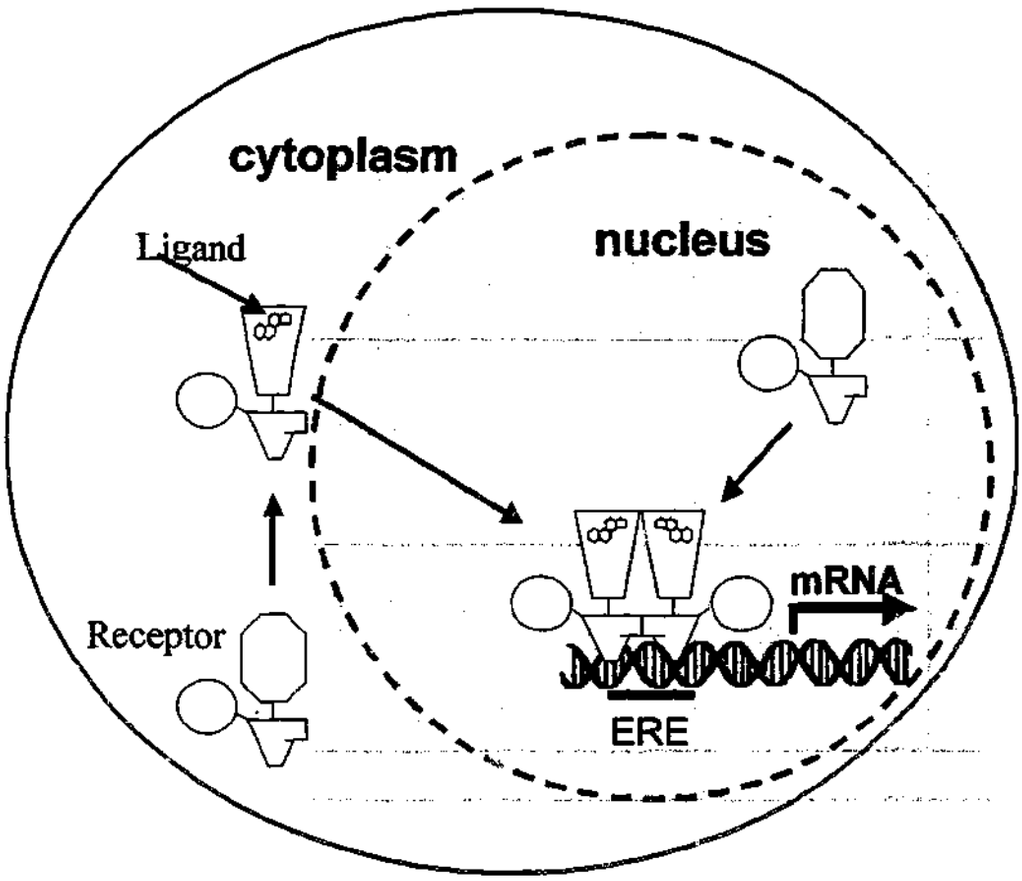


Figure 1.5: A schematic representation of estrogen actions.

Estrogen (ligand) binds to the estrogen receptor and this complex then enters the nucleus and mediates transcription of target genes via the estrogen response element (ERE). Figure kindly provided by Dr. Fraser Rogerson, Prince Henry's Institute of Medical Research.

1.223 Ligand-Independent Actions of Estrogen Receptors

Steroid receptors can be activated in the absence of ligand due to modulation by kinase/phosphatase activity. Specifically, the ERs are responsive to growth factors (Weigel & Zhang 1998). Evidence both *in vitro* and *in vivo* shows that epidermal growth factor (EGF) can activate ERs and this action has been shown to be inhibited by the antiestrogen, N-(n-butyl)-11-[3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl]N-methylundecanamide (ICI-164384) (Weigel & Zhang 1998). In addition to EGFs, other growth factors have also been shown to stimulate ERs *in vitro*, these include transforming growth factor α (TGFα), insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-2 (IGF-2) (Newton *et al.* 1994). Dopamine has also been shown to activate ERs in transfected cells and this could be inhibited by ICI-164384 (Smith *et al.* 1993).

1.23 Mutations in aromatase and ER in humans

Mutations in either the *CYP19* gene or the ERα gene have been identified in humans. Both conditions result in estrogen deficiency. To date, 12 patients with mutations in the *CYP*19 gene have been reported, six females (Harada *et al.* 1992; Ito *et al.* 1993; Morishima *et al.* 1995; Mullis *et al.* 1997; Ludwig *et al.* 1998) and six males (Morishima *et al.* 1995; Carani *et al.* 1997; Deladoey *et al.* 1999; Herrmann *et al.* 2002; Rochira *et al.* 2002a; Maffei *et al.* 2003; Pura *et al.* 2003) and one ERα deficient male (Smith *et al.* 1994). Estrogen deficiency in females leads to a failure of secondary sexual characteristics developing at puberty and estrogen replacement is commenced. Because of this, to date, no adult estrogen deficient females have been characterised. At puberty however, there is a phenotype present in estrogen deficient females due to estrogen deficiency. This includes hypergonadotrophism,

hypogonadism, and presentation with polycystic ovaries. Additionally estrogen deficient females have delayed skeletal maturation. No disruption to lipid homeostasis has been reported, possibly due to the estrogen replacement. Men with aromatase mutations undergo normal puberty, hence estrogen deficient men generally mature with undetected symptoms until adulthood. All estrogen deficient men present with many abnormalities due to estrogen insufficiency such as delayed bone age and tall stature due to a failure of epipyseal fusion (Rochira *et al.* 2002b). A reproductive phenotype has been observed in one patient who presented with oligozoospermia, however his brother who did not have a dusruption to the *CYP19* gene was also azoospermia and therefore this condition may not related to estrogen deficiency (Carani *et al.* 1997). For the purposes of this thesis the focus will be on the disruption to lipid metabolism. For further information refer to (Rochira *et al.* 2002b).

The mutations in the *CYP19* gene of the male aromatase deficient patients have been found in different exons: exon III, exon V, exon VI, two in exon IX and exon X. All mutations result in the abolition of aromatase activity. Due to the absence of estrogen, these men also present with alterations in gonadotropins and androgen levels although the changes are not consistent. One patient presented with elevated serum FSH levels, while serum LH and testosterone were within the normal range (Maffei *et al.* 2003), whereas another patient presented with elevated serum FSH, LH and testosterone levels (Morishima *et al.* 1995). Two of the patients presented with high serum FSH and testosterone, whereas their serum LH levels were normal. The ERα deficient patient also has disrupted hormone levels, however different to those of the aromatase deficient patients. He displayed elevations in serum estradiol and estrone, as well as FSH and LH, whereas serum testosterone concentrations were normal (Smith *et al.* 1994).

All male adult estrogen deficient patients present with lipid abnormalities, but there are some differences in the details. The adult aromatase deficient patients have impaired lipid metabolism as indicated by elevated triglycerides, LDL and total cholesterol, whilst HDL levels are low (Morishima *et al.* 1995; Carani *et al.* 1997; Bilezikian *et al.* 1998; Herrmann *et al.* 2002; Rochira *et al.* 2002a; Rochira *et al.* 2002b; Maffei *et al.* 2003; Pura *et al.* 2003). Conversely, the ERα deficient patient presented with lowered levels of LDL, HDL and total cholesterol and the triglyceride levels were normal (Smith *et al.* 1994). One of the aromatase deficient patients was shown to present with hepatic steatosis and diabetes type 2 however these conditions were reversed by estradiol replacement (Maffei *et al.* 2003). The ERα deficient patient and two of the aromatase deficient patients were also shown to present with hyperinsulinemia (Rochira *et al.* 2002a). These patients clearly show that estrogen is required for a normal lipid profile, however the mechanisms are yet to be fully understood.

1.24 ER Mouse Knockout Models

To further understand the physiological role of estrogen, mouse models of estrogen deficiency have been generated. Knockouts of the two estrogen receptors, αERKO and βERKO; a cross of these resulting in a double knockout, αβERKO and three separate aromatase knockout models, the ArKO, have been genetically engineered.

1.241 Estrogen Receptor alpha knockout mouse, (αERKO)

In 1993 an estrogen receptor null mouse was generated, to the only known estrogen receptor at the time, now known as ERα. A construct containing the neomycin resistance (Neo) and thymidine kinase (TK) sequences was inserted into exon 2 of the ER gene, thereby disrupting the reading frame. Both male and female offspring survived to adulthood and had normal external phenotypes (Lubahn *et al.* 1993). Closer analysis revealed that the female mice were

infertile, having hypoplastic uteri and ovaries which lack corpora lutea, indicating they did not ovulate. Male mice have low testis weight and although sperm was present in their testis and the epididymus, they had a low sperm count. Serum estradiol and LH levels were significantly elevated in knockout females, no other changes were seen in serum FSH and testosterone levels. No changes in serum hormone levels were seen in the αERKO males (Couse & Korach 1999). It was later observed that as the αERKO aged they developed obesity (Heine *et al.* 2000; Ohlsson *et al.* 2000). This was associated with increased adipose tissue in the epididymal, perirenal and inguinal regions. Increases in adipocyte tissue were due to enlarged adipocytes and increased numbers (Heine *et al.* 2000), additionally, there were elevated serum leptin levels (Ohlsson *et al.* 2000). Hyperphagia was not the cause of the obesity, as food intake in the αERKOs was equal to wildtype (WT) controls, however physical energy expenditure may have impacted on the obese phenotype as physical activity was decreased in the αERKOs. A reduction in estrogen signalling also led to impairments in glucose metabolism as indicated by insulin resistance and reduced glucose tolerance (Heine *et al.* 2000). Additionally, there were disruptions to serum cholesterol levels, which were elevated compared to controls and smaller LDL particles (Ohlsson *et al.* 2000). This knockout model demonstrates that estrogen is important in the regulation of obesity and lipid homeostasis, particularly when signalling through ERα.

1.242 Estrogen Receptor beta knockout mouse (βERKO)

A second estrogen receptor was cloned in 1996 (Kuiper *et al.* 1996), therefore to understand the functions of this receptor, an ERβ knockout model was generated. Exon 3 of the ERβ gene was disrupted by inserting a neomycin-resistant gene (Neo) in the reverse orientation (Krege *et al.* 1998). The phenotype of the βERKO differed from the αERKOs, namely both

males and females were fertile although the female mice produced fewer and smaller litters, most likely due to atresia of more immature follicles and fewer corpora lutea. Histological examination of the urogenital tract of 3-4 month old male mice revealed no obvious abnormalities (Krege *et al.* 1998). In addition there were no changes in serum hormone levels (Couse *et al.* 2003). Unlike the αERKO the βERKO did not display an obese phenotype nor did it have any alterations in serum cholesterol profile (Krege *et al.* 1998). This indicated that the ERα is the more important receptor in relation to regulation of obesity and lipid homeostasis by estrogens.

1.243 Double Estrogen Receptor Knockout Mouse (αβERKO)

Crossing of βERKO and αERKO mice generated the αβERKO mice. Adult αβERKO female mice exhibit ovarian follicles which resemble seminiferous tubules from the testis, including Sertoli-like cells. Hence loss of both receptors leads to an ovarian phenotype that is distinct from the individual ERKOs (Couse *et al.* 1999). The male αβERKO presented with a similar phenotype to the αERKO males (Dupont *et al.* 2000). Serum hormone levels revealed elevations in LH and testosterone (Couse *et al.* 2003). Additionally the αβERKO displayed the same obese phenotype as the αERKO (Krege *et al.* 1998), again indicating a role for estrogen in regulating factors relating to obesity and lipid homeostasis which is mediated through the ERα and not the ERβ.

1.244 Limitations to the Estrogen Receptor Knockout Models

A major limitation of the ER knockout models is that the phenotype cannot be reversed. An estrogen deficient model, that lacks circulating estrogens but still has intact ERs, is able to

have estrogen replaced in an attempt to reverse any phenotypes, making this a more suitable model to study the roles estrogen *in vivo*.

A secondary problem recently identified in the ER knockout models, is some residual activity of the ERα. The technology used to generate the knockout models was insertion of the neo gene. This still enabled alternative splicing of the ER mRNA and a 61KDa ERα protein variant has been found in the αERKO and its activity has been shown to be as high as 75% of WT ERα (Kos *et al.* 2002a; Kos *et al.* 2002b). As both the αERKO and βERKO were generated using the same technology, it is possible that the βERKO also has residual ERβ activity. To address this problem Dupont and colleagues have generated compound knockouts of both receptor subtypes and a double knockout mouse (Dupont *et al.* 2000). No obese phenotype has been described as yet for these models.

1.25 The Aromatase Knockout (ArKO) Mouse Model

1.251 Generation of the ArKO Mouse

As stipulated above, a mouse model totally devoid of circulating estrogens would allow the study of the physiological effects of estrogen in both male and female mice. Additionally, in such a model, estrogen could be replaced to reverse the effects of the absence of estrogen.

In 1998 (Fisher *et al.* 1998) generated an ArKO, which is totally devoid of endogenous estrogens. Exon IX of the gene was the chosen site for disruption, as the coding sequence between *Eco*RV (bp 1047) and *Xho*I (bp 1210) is highly conserved in all species so far examined (Simpson *et al.* 1997a). The knockout mouse was generated with the insertion of the target neomycin cassette in exon IX of the *Cyp19*, deleting 163bp of this exon, thus resulting in a truncated protein (Figure 1.6, reproduced from Fisher *et al.* 1998). Aromatase activity was assessed from ovaries 12 week old ArKO and WT using the tritiated water

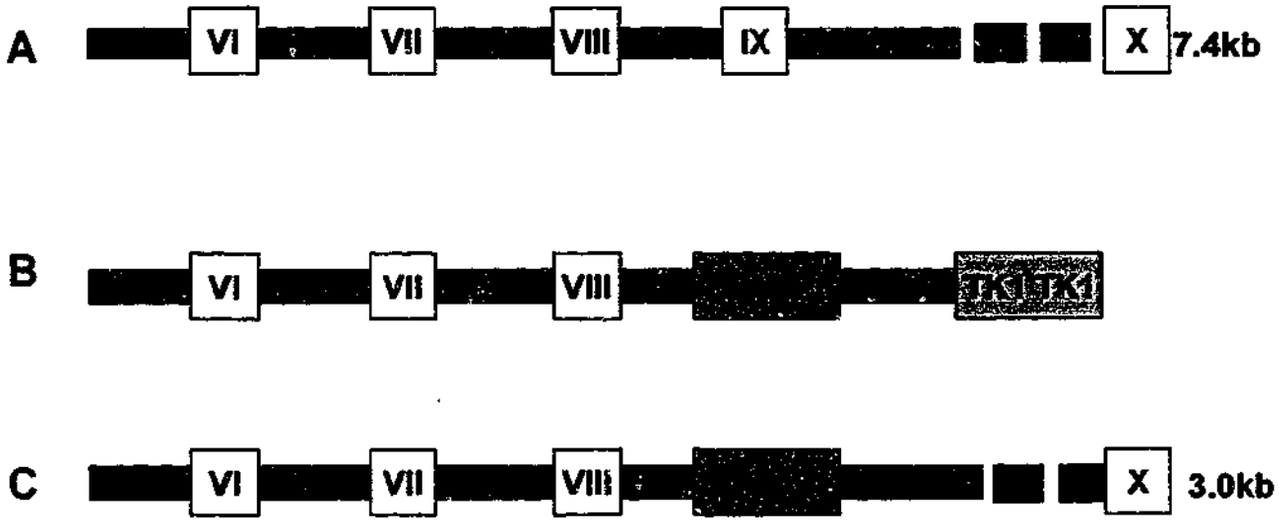


Figure 1.6: Schematic representation of a segment of the mouse *Cyp19* gene.
A Represents WT fragment of 7.4kb. **B** Represents targeting vector in exon XI and **C** Represents disrupted exon IX, 3.0kb fragment. Neo, refers to the neomycin resistant cassette. TK1 refers to the two herpes simplex virus thymidine kinase genes type 1. Roman numbers refer to exon number.

release assay (Ackerman *et al.* 1981). The ovaries from the ArKO showed no activity, therefore the disruption of the aromatase gene was successful (Fisher *et al.* 1998). The ArKO mouse thereby provides us with an estrogen deficient mouse model ideal to study the actions of estrogen *in vivo* in both males and females.

Two other groups have also generated aromatase deficient mice (Honda *et al.* 1998; Nemoto *et al.* 2000). Nemoto and colleges also generated their model by replacing 87 base pairs of exon IX with a neo gene, which was derived from pMC1-neo. This model also lacks a function aromatase enzyme and hence has no circulating estrogens (Nemoto *et al.* 2000).

In 1998, another group also generated an aromatase deficient mouse but they did by disrupting exons I and II of the *Cyp19* gene (Honda *et al.* 1998). Briefly, a *Sph*I fragment (2.9kb) which included exons I and II as well as the proximal promoter region was deleted by the insertion of neo. This disruption also led a nonfunctional aromatase enzyme, hence an estrogen deficient model was generated.

This section of the literature review will focus on the phenotypes of the estrogen deficient mice. The Fisher model, our model, has been the most extensively studied, however some studies have been performed in the other models. Female ArKO mice presented with serum testosterone levels 10 times that of WTs (Fisher *et al.* 1998; Britt *et al.* 2000). Similarly ArKO male mice had elevated levels, although variable (Fisher *et al.* 1998; Robertson *et al.* 1999). Additionally, gonadotrophin levels were also altered in these mice. While estrogen deficient females had elevated LH and FSH levels compared with WT (Fisher *et al.* 1998; Britt *et al.* 2000), estrogen deficient males exhibited elevated LH levels (Fisher *et al.* 1998; Robertson *et al.* 1999) but unchanged FSH levels (Robertson *et al.* 1999). Examination of these mice revealed that estrogen deficiency leads to a block in folliculogenesis and an

atrophic uterus (Britt *et al.* 2000; Toda *et al.* 2001c). In the ovaries of estrogen deficient female mice there was also the development of sertoli-like cells (Britt *et al.* 2002), normally present only in male testis. A disruption to reproduction in the males was also observed with a block in spermatogenesis at the round spermatid stage, seen as early as 18 weeks in some ArKO mice, however by one year of age all ArKO mice presented with this phenotype (Robertson *et al.* 1999). In addition to disrupted spermatogenesis, lack of sexual behaviour has also been observed in the ArKO male mice (Robertson *et al.* 2001; Toda *et al.* 2001a). This is restored in 90% of ArKO mice following estrogen replacement from birth (Toda *et al.* 2001a). Hyperplasia has also been observed in the prostate (McPherson *et al.* 2001).

Interestingly, sexually dimorphic phenotypes have been observed in various tissues of the estrogen deficient mice. Estrogen deficient females have increased bone turnover, whereas the males have decreased osteoblastic and osteoclastic surfaces, revealing sexual dimorphism in bone formation (Oz *et al.* 2000). Additionally, in the brain, while apoptosis in ArKO male mice occurs in the arcuate nucleus (Arc) and medial preoptic area (MOP) regions of the hypothalamus and pons regions (Hill *et al.* 2003), in the female ArKO mice cell death has been observed in the frontal cortex of the brain (Boon *et al.* 2002). Disruption to metabolic processes has also been characterised in these models and will be described in greater detail in the next Section, 1.252. For the purposes of this thesis the focus will be on the disruptions to the metabolic phenotype.

1.252 The Metabolic Phenotype

Metabolic phenotypes have been described in two of the ArKO models (Fisher *et al.* 1998; Nemoto *et al.* 2000). Estrogen deficiency in both males and females results in age progressive onset of obesity (Jones *et al.* 2000; Takeda *et al.* 2003). From as early as three months of age, both gonadal and infrarenal fat depots were significantly elevated in a state of

estrogen deficiency most likely due to increased adipocyte volume. Estrogen (E_2) replacement was able to reverse this (Jones *et al.* 2000; Misso *et al.* 2003). Increases in fat pad masses correlated with increases in circulating leptin levels (Jones *et al.* 2000) as well as elevated leptin transcripts, again this could be reversed with E_2 replacement (Misso *et al.* 2003). In addition to increased adiposity, there was a significant decrease in lean body mass. This may be attributed in part to a decrease in physical activity, which was seen in older female ArKO mice. A similar trend was observed in younger female ArKO mice, however it was not statically significant (Jones *et al.* 2000). Hyperphagia was not the cause of obesity, as a food intake study revealed equal feeding between genotypes in both models (Jones *et al.* 2000; Takeda *et al.* 2003). Another factor leading to the increase in adiposity may be an elevation in lipoprotein lipase (LPL) expression (Misso *et al.* 2003). This may be increasing the hydrolysis of serum triglycerides releasing free fatty acids (FFA) and sn2-monoglycerides, which are taken up by the adipose tissue (Fredrikson & Belfrage 1983). Estrogen deficiency did not appear to affect hormone sensitive lipase (HSL) expression (Misso *et al.* 2003), which is responsible for catalysing hydrolysis of intracellular triglycerides for release of FFA (Lewis *et al.* 2002). However, HSL is regulated primarily at the level of the catalytic activity via a cAMP-dependent mechanism (Okuda *et al.* 1994; Morimoto *et al.* 2001), therefore there may be changes occurring posttranscriptionally. Additionally, β-oxidation rates were unchanged in gonadal fat depots (Misso *et al.* 2003). As well as disruptions to lipid metabolism, glucose oxidation rates were decreased by 59% in estrogen deficient females (Jones *et al.* 2000). At six months of age estrogen deficiency led to an elevation in fasting blood glucose levels and at 18 weeks the animals had severe impairment in glucose tolerance, which correlated with a reduced response to insulin (Takeda *et al.* 2003). E_2 replacement from birth improved glucose tolerance and insulin sensitivity, as

did E_2 replacement from 24 weeks for a 12 week period (Takeda *et al.* 2003), indicating that estrogen mediation of glucose homeostasis is not programmed early in rodents.

Serum lipid profiles were also examined and estrogen deficiency in one year old mice led to elevations in serum cholesterol and HDL levels in both sexes, however only males had elevated serum triglyceride levels (Jones *et al.* 2000). Gender differences were also observed at 3-4 months in the estrogen-replete mice, namely females had significantly lower cholesterol levels compared with males. A similar result was observed comparing estrogen deficient females and males although it did not reach significance. Also at this age, females had significantly lower levels of triglycerides compared to males, whereas the reverse was seen for HDLs, namely females had elevated levels compared with males (Jones *et al.* 2000). These results clearly show that estrogen is an important player in regulating lipid homeostasis in males and females.

Estrogen deficiency also led to hepatic steatosis, fatty liver, in older mice (Jones *et al.* 2000; Nemoto *et al.* 2000). Studies performed in the third ArKO model (Nemoto *et al.* 2000) reveal by Northern blot analysis revealed that there were decreases in very long chain fatty acyl-CoA synthetase (VLACS) and peroxisomal acyl-CoA oxidase (AOX), enzymes that are involved in the first two steps of peroxisomal β-oxidation. Additionally, medium-chain acyl-CoA dehydrogenase (MCAD), the rate-limiting step in the mitochondrial pathway of medium chain oxidation was also decreased, indicating both the peroxisomal and mitochondrial pathways were disrupted (Nemoto *et al.* 2000). This impairment was also seen for fatty acid β-oxidation activity in both pathways. Using tetracosanoic acid (C24:0) as a substrate to measure peroxisomal activity and palmitic acid (C16:0) and lauric acid (C12:0) to measure mitochondrial activity, all were reduced in the absence of estrogen (Nemoto *et al.* 2000). The ArKO mice were treated with $E_2$from birth until six months of age and this was able to

reverse the hepatic steatosis seen in their livers and increased fatty acid β-oxidation at the level of transcripts and activity (Nemoto *et al.* 2000). Also serum very low-density lipoprotein (VLDL) was measured and was the same regardless of estrogen status (Toda *et al.* 2001b), indicating that export of triglycerides out of the liver is similar in both genotypes, hence probably not a contributing factor in the development of hepatic steatosis. This data further strengthens the hypothesis that estrogen plays an important role in lipid and carbohydrate homeostasis.

Peroxisome proliferator-activated receptor α (PPARα) is a member of the nuclear receptor superfamily of transcription factors and is believed to be an important regulator of fatty acid oxidation (Djouadi *et al.* 1998). It enhances transcription of target genes by binding to a peroxisomal proliferator response element (PPRE) on target sequences. The ability of PPARα to bind to this element was not effected by estrogen status (Nemoto *et al.* 2000), indicating this is not the mechanism by which estrogen regulates fatty acid (FA) metabolism.

The second ArKO mouse model (Honda *et al.* 1998) has yet not reported a metabolic phenotype, but they have reported a disruption to hepatic expression of certain cytochrome P450s. Specifically, the researchers have examined the members of the *Cyp1-3* families of enzymes which are known to metabolise a variety of compounds to inactive products (Yamada *et al.* 2002). The authors found that the absence of estrogen led to a decrease in *Cyp3a11* protein expression in both males and females. Additionally, they found that the female-specific *Cyp3a41* was undetectable in the absence of estrogen and this could not be reversed by E_2 administration to adults, indicating neonatal programming or infantile exposure to estrogen. Also expression of the female-specific *Cyp2d9* was absent in estrogen deficient mice. Expression of *Cyp3a41* on the other hand, was restored in some mice with E_2

administration (Yamada *et al.* 2002). These results indicate an important role for estrogen in regulating specific expression of P450s in the rodent liver.

1.3 Fatty Acids and triglycerides

The liver has two major sources of fatty acids, *de novo* lipogenesis and fatty acid uptake. Within the liver, fatty acids and triglycerides have three major fates: hepatic storage, oxidation and secretion as VLDL. This section of the literature review will focus on these pathways and their regulation.

1.31 de novo Lipogenesis

De novo synthesis of fatty acids or *de novo* lipogenesis (DNL) occurs within the cytoplasm of cells and the precursor for this process is acetyl CoA. Acetyl CoA is the product of fatty acid oxidation and glycolysis (described in Section 1.32) and these processes mainly occur in the mitochondria of cells. Therefore in order for DNL to occur, acetyl-CoA needs to be transported out of the mitochondria and into the cytosol. To be transported firstly, acetyl-CoA must be converted to citrate by citrate synthase and transported via the citrate transporter. Once in the cytoplasm, cleavage occurs, catalysed by adenosine triphophate (ATP):citrate lyase, producing acetyl CoA and oxaloacetate. During this process citrate also activates acetyl-CoA carboxylase α (ACCα) (Lane *et al.* 1974; Volpe & Vagelos 1976), which is the first enzyme in DNL and produces malonyl CoA. In addition to malonyl CoA's role in DNL, malonyl CoA also acts to inhibit mitochondrial β-oxidation by inhibiting carnitine palmitoyl transferase I (CTPI) (McGarry *et al.* 1977).

From this point, the overall production of fatty acids occurs under the regulation of fatty acid synthase (FAS), which is a multifunctional complex made up of six enzymes (Kumar *et al.*

1970; Bratcher & Hsu 1976). The centre of the complex is acyl carrier protein (ACP), which was first identified from *E. coli* in 1988 (Holak *et al.* 1988). FAS contains a prosthetic group, known as 4'-phosphopantotheine which is covalently linked to the ACP and its SH group is esterified to the malonyl group during fatty acid synthesis. All of the enzymes in the FAS complex participate in the elongation reaction where fatty acid production occurs from one acetyl-CoA and seven malonyl-CoA and through a series of reactions consisting of a condensation reaction followed by a reduction, then a dehydration step and finally another reduction reaction. These reduction reactions employ NADPH as the source of reducing equivalent. This process repeats itself until the formation of the 16 carbon palmitate.

This reaction can be summarised as:

$$\text{Acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\text{NADPH} + 14\text{H}^{+} \rightarrow$$

$$\text{palmitate} + 8\text{CoA} + 6\text{H}_2\text{O} + 7\text{ADP} + 7\text{P}_i + 14\text{NADP}^{+}.$$

(Further detail on this process is reviewed in (Smith *et al.* 2003).)

1.32 Fatty acid β-oxidation

The process of β-oxidation is the sequential removal of two carbons from FA eventually ending with acetyl-CoA, the reverse of DNL. Electrons which are generated in the process pass through the respiratory chain, which drives ATP synthesis. Additionally, acetyl-CoA is completely oxidised to CO_2 in the citric acid cycle, thus conserving energy. β-oxidation

occurs in primarily the mitochondria however it also occurs in the peroxisomes. This section will provide a brief overview of the two pathways of β-oxidation, the mitochondrial and the peroxisomal (Figure 1.7, reproduced from Nelson & Cox 2000).

1.321 Mitochondrial pathway

Most FA β-oxidation occurs in the mitochondria. FA delivery is via different mechanisms depending on their length. Delivery of short chain (SCFA) (C_4 to C_6) and medium chain (MCFA) (C_8 to C_{12}) fatty acids occurs by simple diffusion across the mitochondrial membrane into the mitochondrial matrix, where they are esterified. Esterified LCFA are transported across the plasma membrane by the carnitine cycle shuttle mechanism. Firstly, LCFA esters must be coupled to carnitine; this reaction is catalysed by carnitine palmitoyltransferase I (CPTI) and once inside the inner mitochondrial matrix, the acyl-carnitine is then converted back to LCFA esters and this reaction is catalysed by carnitine palmitoyltransferase II (CPT2) (Sim *et al.* 2002). From this point, SCFA-CoA, MCFA-CoA and LCFA-CoA, are able to undergo the repeated four-step process of β-oxidation.

As with the building of fatty acids, by two carbons at a time (described in Section 1.31), β-oxidation involves removal of two carbons at a time, forming acetyl-CoA. This removal begins at the carboxyl end of the fatty acid chain, with four enzymes participating in this process. Firstly, a flavoprotein-linked dehydrogenation step begins, which is catalysed by acyl-CoA dehydrogenase (ACD), secondly a dehydration step follows, catalysed by enoyl-CoA hydratase (ECH), thirdly is a hydrogenase step, linking NAD^+, β-hydroxyacyl-CoA dehydrogenase catalyses this reaction. The final reaction in this process is the thiolytic cleavage by acyl-CoA acetyltransferase (thiolase) resulting in the acetyl-CoA plus acyl-CoA (Sim *et al.* 2002). The equation for one cycle beginning with palmitoyl-CoA is:

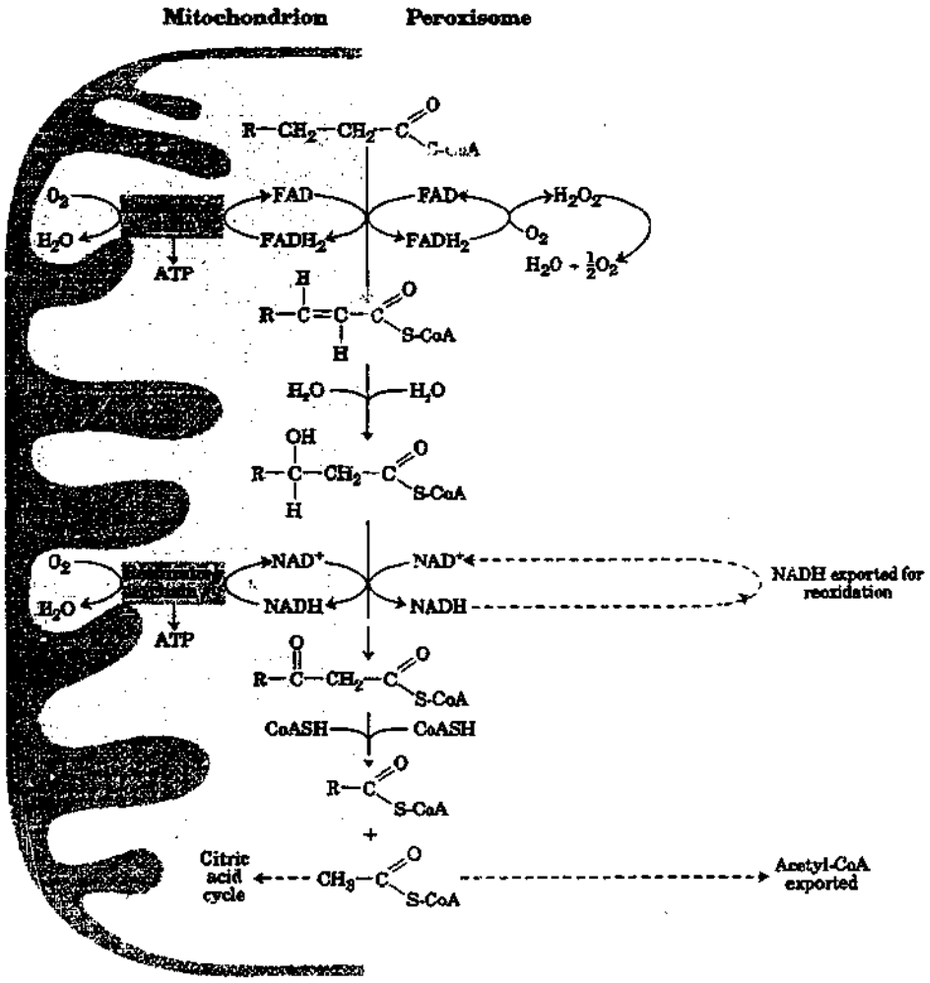


Figure 1.7: Comparison of the mitochondrial and peroxisomal fatty acid β-oxidation

The first dehydrogenation step differs, in the mitochondrial pathway electrons generated move into the respiratory chain and in the peroxisomal pathway there is the production of H_2O_2. Secondly, acetyl-CoA produced in the peroxisomes does not directly enter the citric acid cycle, as it does in the mitochondrial pathway.

$$\text{Palmitoyl-CoA} + \text{CoA} + \text{FAD} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow$$

$$\text{acyl-CoA} + \text{acetyl-CoA} + \text{FADH}_2 + \text{NADH} + \text{H}^+$$

The second stage in fatty acid β-oxidation involves the newly formed acetyl-CoA entering the citric acid cycle and being oxidised to CO_2. Finally step is the electrons generated via stages one and two are then able to enter the respiratory chain (electron transfer) and thereby producing ATP from oxidative phosphorylation .

1.322 Peroxisomal pathway

In addition to mitochondrial β-oxidation, the peroxisomes are also able to perform this process but to a lesser extent. This process differs somewhat from the mitochondrial pathway. Peroxisomes are cell organelles and their primary role is in hydrogen peroxide metabolism (De Duve & Baudhuin 1966). As in the mitochondrial pathway, before β-oxidation can occur in the peroxisomal pathway, FA must undergo esterification. LCFA are converted to the acyl-CoA derivative by long-chain acyl-CoA synthetase (Shindo & Hashimoto 1978; Mannaerts *et al.* 1982) and VLCFA are converted by very long-chain fatty acid synthetase (Singh & Poulos 1988; Lazo *et al.* 1990). The long-chain acyl-CoA synthetases can be found in both the mitochondrial and peroxisomal compartments but only the peroxisomes have the very long-chain acyl-CoA synthetase (Singh & Poulos 1988). Another difference between the mitochondrial and peroxisomal pathways is that the LCFA do not require the carnitine transport system for export into the peroxisome (Thomas *et al.* 1980; Hertz & Bar-Tana 1992).

Like the mitochondrial pathway there are four main reactions in the process of β-oxidation and this occurs in the peroxisomal matrix. Firstly, is the dehydrogenation step, which differs from the mitochondrialpathway whereby instead of electrons moving into the respiratory chain H_2O_2 is produced. The second reaction is the hydration reaction, followed by the formation of 3-ketoacyl-CoA and the last step is the thiolytic cleavage leaving the FA two carbons shorter, by the release of acetyl-CoA and an acyl-CoA. This process repeats itself (Lazarow 1978; Reddy & Mannaerts 1994). Also the acetyl-CoA produced in the peroxisomes does not directly enter the citric acid cycle due to the lack of enzymes present in this structure and therefore this acetyl-CoA may move into the mitochondria to be further oxidised.

1.36 Regulation of fatty acid pathways

Due to the serious health consequences of over production and/or under clearance of lipids from the body, factors that regulate fatty acids are highly important. Several transcription factors have been implicated in the regulation of fatty acids. These include the sterol regulatory element binding proteins (SREBP), liver X receptor (LXR) and peroxisomal proliferator-activated receptors (PPAR). SREBPs have been shown to regulate genes involved in lipogenesis by regulating FAS, ACCα, glycerol-3-phosphate acyltransferase and LPL (Schoonjans *et al.* 2000). The FAS promoter region contains a liver X receptor-response element (LXR-RE). Additionally, the expression of FAS was altered in LXRα deficient mice, indicating that LXRα may regulate fatty acid synthesis, but the exact mechanisms are yet to be elucidated (Schoonjans *et al.* 2000).

PPAR have also been implicated in the regulation of FA. There are three subtypes, designated PPARα, PPARγ and PPARδ/β and they have different tissue distribution and their

regulation of fatty acids differs in these tissues. Structurally the three subtypes are similar, containing a central DNA-binding domain, which has two zinc-fingers and a ligand-binding domain present in the C-Terminus. Many studies have shown that FA are endogenous ligands for the PPARs (Forman *et al.* 1995; Kliewer *et al.* 1995; Krey *et al.* 1997; Kliewer *et al.* 1997; Forman *et al.* 1997a). PPARα is the subtype present in the liver, heart, muscle and kidney. Its role is in the regulation of FA catabolism (Dreyer *et al.* 1993; Peters *et al.* 1997). Specifically, PPARα has been shown to regulate the gene encoding acyl-CoA oxidase (ACO), which catalyzes the rate-limiting step in the peroxisomal β-oxidation of fatty acids. Within the promoter of the ACO gene is a peroxisome proliferator response element (PPRE), which is where PPARs bind (Dreyer *et al.* 1993). Additionally, PPARα also regulates FA binding proteins (FATP) and ω-oxidation (Lee *et al.* 2003), as well as down regulating apolipoprotein C-II, which is involved in the inhibition of triglyceride hydrolysis by LPL (described in Section 1.421) (Lee *et al.* 2003). PPARγ is expressed mainly in adipocytes and macrophages, regulating adipocyte differentiation, lipid storage and glucose homeostasis (Barak *et al.* 1999; Kubota *et al.* 1999; Rosen *et al.* 1999). PPARδ is ubiquitously expressed and has been suggested to play a role in lipid homeostasis as that of a sensor for triglycerides present in VLDL. The activation of PPARδ by VLDL has been shown to induce transcription of adipocyte differentiated regulating protein (ADRP) (Chawla *et al.* 2003). Therefore the PPARs play an important role in regulating FA.

1.37 Fatty Acid Transporters

There are two known mechanisms of FA transport, passive diffusion and protein-facilitated transport. Passive diffusion involves the flip-flop method for movement of FA through the lipid bilayer, but due to low rates of diffusion the method would not allow enough FA to

across the membrane for biological function be performed (Berk & Stump 1999; Kleinfeld 2000). Therefore the second process known as protein-facilitated transfer must be highly signifcant.

A family of proteins known as fatty acid transporter proteins (FATPs) has recently been discovered and they participate in the transfer of LCFA (Schaffer & Lodish 1994). Six FATPs have been identified, designated FATP1-6 (Stahl *et al.* 2001). The FATPs are integral transmembrane proteins with a structure similar to long-chain acyl-CoA synthetase. They contain a highly conserved domain which is related to ATP-binding and hydrolysis. This domain appears important for FA transport but the mechanisms are yet to be fully elucidated.

In addition to the FATP other proteins have also been implicated in LCFA transport: ADRP, caveolin (Gao & Serrero 1999; Serrero *et al.* 2000; Razani *et al.* 2002) and CD36/FAT. CD36/FAT is the most extensively studied transporter and it belongs to the scavenger receptor class B type I (SR-B1) family of scavenger receptors (Stahl *et al.* 2001). CD36/FAT is an integral membrane glycoprotein, which is expressed at the cell surface and within lysosmes. Furthermore it is localised within membrane rafts and is present in a wide variety of tissues (Hajri & Abumrad 2002). ADRP has been shown to localise to neutral lipid storage droplets in a wide variety of cells, suggesting that it plays a role in the management of neutral lipid stores (Brasaemle *et al.* 1997).

Insulin, cytokines (Memon *et al.* 1998) and PPAR ligands (Martin *et al.* 1997; Motojima *et al.* 1998; Martin *et al.* 2000) have all been shown to regulate the FA transporters. Furthermore the FATP1 promoter region has been shown to contain a PPAR binding element (Hajri & Abumrad 2002). These transporters are present in a variety of tissues but of particular importance to this thesis, are the transporters which are present in the liver, including,

CD36/FAT, FATP2 (Hirsch *et al.* 1998), FATP3, FATP5 (Fitscher *et al.* 1998) and ADRP (Jiang & Serrero 1992).

1.36 Triglyceride packaging and export

The liver assembles triglycerides into VLDL particles for secretion. Apolipoprotein B (apoB) is the major lipoprotein present in the VLDL, and it is required for the assembly and secretion of VLDL from the liver. Regulation of apoB occurs posttranscriptionally given that the apoB gene is constitutively expressed and regulation occurs either during its translocation into the endoplasmic reticulum or via its rate of degradation (Adeli *et al.* 2001). Additionally apoE, (Mahley *et al.* 1970; Swift *et al.* 1980) apoC-I, apoC-II and apoC-III (Ginsberg 1998) are also a part of newly synthesised VLDLs. Formation of the VLDL occurs in the endoplasmic reticulum and full maturation occurs in the Golgi apparatus of the hepatocyte before its secreted (Ginsberg 1998). This process is mediated via microsomal triglyceride transfer protein (MTTP), which catalyses the lipid transfer of the apoB polypeptide, in addition to participating in the formation of triglyceride rich droplets in the ER, which are also able to fuse with apoB particles (Gordon & Jamil 2000). MTTP is also required for triglyceride packaging into chylomicrons in the small intestine (described in Section 1.421). The apoCs have important functions on the VLDL surface once the lipoprotein is secreted from the liver. ApoC-II is a cofactor necessary for LPL actions of hydrolysing triglycerides from the VLDL particle for uptake into adipose tissue and muscle. Conversely, apoC-III is believed to inhibit this stimulation of LPL. Once the core triglycerides have been removed, the liver can eliminate the VLDL remnants, also known as IDL. This process involves apoE mediated uptake via receptor mediated pathways. Additionally, they can be converted to LDL and then be removed via the LDL-receptor pathway (described in Section 1.422) (Ginsberg 1998).

1.37 Regulation of these pathways by estrogen

Premenopausal women have lower levels of serum triglycerides compared with men and postmenopausal women, suggesting estrogen plays a role in their regulation. The exact mechanisms of this regulation are not completely understood. Estrogen has an effect on *de novo* synthesis of lipids. In male turkeys, estrogen has been shown to stimulate *de novo* FA synthesis in the liver, which was shown by an increase in $[^3H]H_2O$ incorporation into the phospholipids and triglycerides, present in both the liver and plasma (Dashti *et al.* 1983). Estrogen administration to male *Xenopus laevis* revealed increases in FA synthesis due to increases in acetyl-CoA carboxylase activity (Philipp & Shapiro 1981), although estrogen reduced *de novo* FA synthesis in adipose tissue of ovariectomized ewes (Green *et al.* 1992). Estrogen has been shown to suppress the transcription of LPL, which catalyses hydrolysis of serum triglycerides releasing FFA and sn2-monoglycerides for uptake into the adipose tissue (Fredrikson & Belfrage 1983). In humans, apoE has four different alleles and in postmenopausal women on HRT a favourable lipid profile is associated with specific alleles (Tolosa *et al.* 2001; von Muhlen *et al.* 2002; Lehtimaki *et al.* 2002), indicating estrogen plays a role in lipoprotein physiology. Estrogen may also play a role in regulating β-oxidation as shown in one of the ArKO mouse models (described in Section 1.252) (Nemoto *et al.* 2000). Despite the epidemiological data suggesting estrogen plays a role in triglyceride homeostasis, more research is needed to understand the exact mechanisms and which pathways are regulated by estrogen.

1.4 Cholesterol metabolism

Cholesterol is the precursors for all steroid hormones, integral to the formation of bile acids and an essential component of cellular membranes. Cholesterol can be synthesised from

acetyl-CoA or taken up from mainly from animal products in the diet, and is cleared from the body via the bile acid pathway. Cholesterol synthesis and uptake are very tightly controlled processes. Excess serum LDL cholesterol can be taken up by macrophages causing the formation of foam cells which are then able to form atherogenic plaques, playing a key role in the pathogenesis of cardiovascular disease. Therefore understanding the mechanisms that regulate cholesterol homeostasis will help in the development of suitable cholesterol lowering drugs.

1.41 De novo synthesis of cholesterol

De novo synthesis of cholesterol utilises acetyl-CoA, like FA, but the assembly plan is different (Figure 1.8). *De novo* synthesis of cholesterol occurs in four stages, three molecules of acetyl-CoA condense to form the six carbon compound β-hydroxy-βmethylglutayl- CoA (HMG-CoA), these reactions are catalysed by thiolase and HMG CoA synthase. HMG CoA reductase catalyses the reduction of HMG-CoA to mevalonate, where HMG CoA reductase is a integral membrane protein of the smooth endoplasmic reticulum and also provides the rate-limiting step in cholesterol biosynthesis. Mevalonate is then converted to isoprenes units. Condensation of three activated isoprene molecules leads to the formation of farnesyl pyrophosphate and condensation of two farnesyl units leads to the formation of squalene. This reaction is catalysed by squalene synthase. The final stage is cholesterol biosynthesis is conversion of the linear squalene into the four ring steroid nucleus of cholesterol. This pathway was first discovered by Konrad Bloch in 1965 (Bloch 1965) and is reviewed in Vance & Van den 2000.

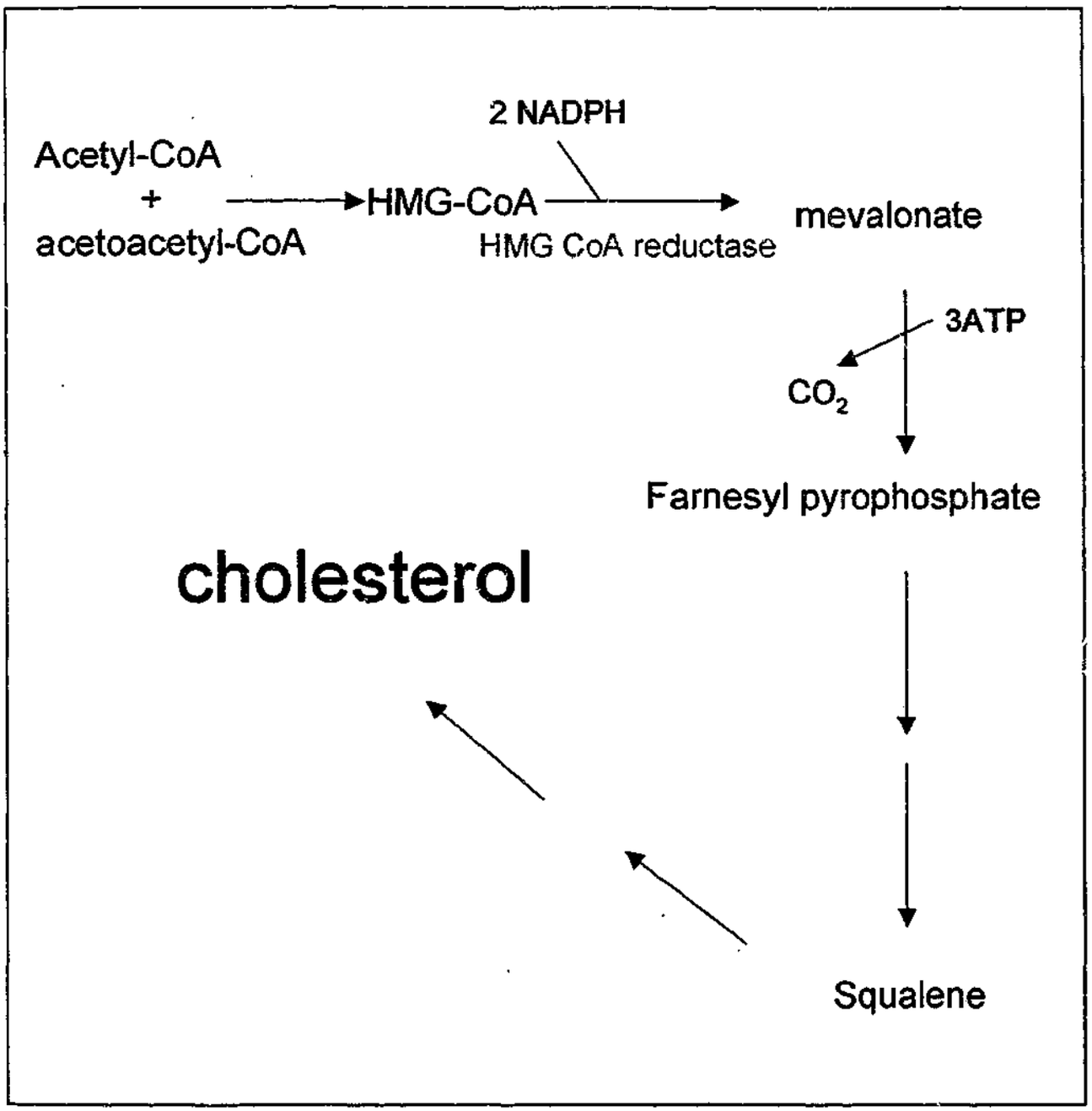


Figure 1.8: Cholesterol Biosynthesis
Firstly there is condensation of acetyl-CoA and acetoacetyl-CoA produces HMG-CoA. This then is reduced to mevalonate and the reaction is catalysed by HMG CoA reductase the rate-limiting step in this reaction. Mevalonate is then converted to two activated isoprenes. Following this there is condensation of six activated isoprene molecules leads to the formation squalene. The final stage is cholesterol biosynthesis is the linear squalene being converted into the four ring steroid nucleus of cholesterol.

1.42 Cholesterol Uptake

Due to the hydrophobic nature of cholesterol esters and triglycerides, their transport through the body via blood vessels is facilitated by specialised particles known as lipoproteins (Ginsberg 1998). Lipoproteins are comprised of a core made up of the hydrophobic cholesterol esters and triglycerides, surrounded by an amphipathic monolayer of phospholipids, free cholesterol and proteins, apoproteins (apo), apos they are important regulators of lipid transport (Ginsberg 1998). There are four major classes of lipoproteins and their names reflect their buoyant densities (Krieger M. 1999). Dietary cholesterol is absorbed in the small intestines and packaged into chylomicrons where it can be transported to the liver (described in Section 1.421). The LDL is the major cholesterol carrying lipoprotein (described in Section 1.422), and high levels in plasma are correlated with increased atherosclerosis. HDL is involved in reverse transport of cholesterol, (described in Section 1.423) and is associated with a decreased risk of atherosclerosis. VLDL principally carries triglycerides that are synthesised in the liver, (described in Section 1.36)(Figure 1.9 reproduced from Nelson & Cox 2000).

1.421 Uptake of dietary cholesterol: chylomicrons

Dietary cholesterol is absorbed in the small intestines and packaged into chylomicrons where it can be transported throughout the body. AcylCoA:cholesterol acyltransferase 2 (ACAT2) converts cholesterol to cholesterol ester, and this is packaged into chylomicrons, this esterification of cholesterol by ACAT2 also occurs within the liver (Buhman *et al.* 2000). The lipoproteins which predominantly make up chylomicrons, are apo B-48, apo A-I, apo-A-II and apo-IV and phospholipids are also present (Ginsberg 1998). After the chylomicra enter the plasma, they gain apo C-I, apo C-II , apo C-III and apo E, which they obtain from the

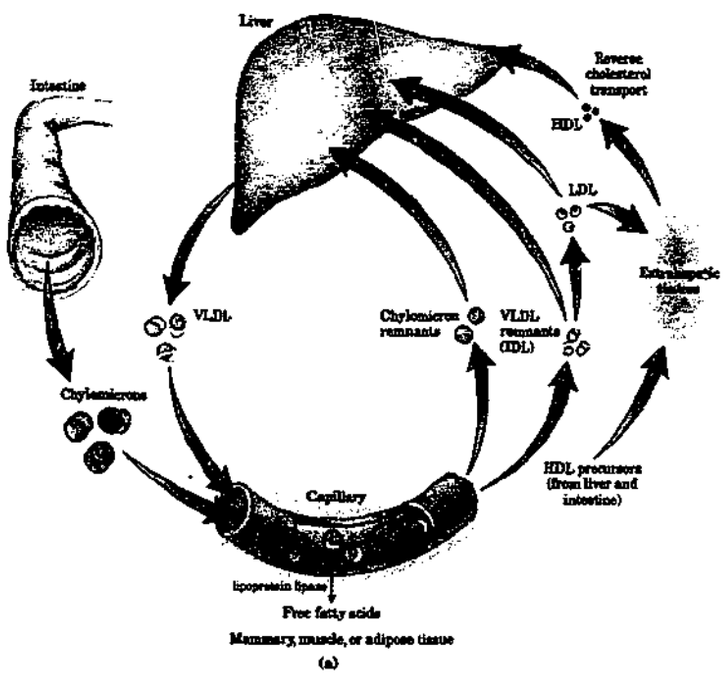


Figure 1.9: Lipid transport and Lipoproteins
Lipids are transported in lipoproteins. Triglycerides and cholesterol are packaged into chylomicrons in the small intestine. Lipids, mainly triglycerides can be packaged in very low-density lipoproteins (VLDL) and exported out of the liver. The triglyceride component is released to adipose tissue or muscle through the actions of lipoprotein lipase (LPL), the chylomicrons remnants are taken up by the liver. The VLDL remnants (IDL) can either be endocytosed by the liver or be converted to LDL, which can be taken up by the liver or internalised by extra hepatic tissues. Cholesterol can be removed from these tissues via reverse cholesterol transport in high-density lipoproteins (HDL). (Figure reproduced from Nelson and Cox, 2000)

surface of HDL. At this time there is free transfer of cholesterol, cholesterol esters and phospholipids to the HDL (Ginsberg 1998). The function of apo C-II is to activate lipoprotein lipase (LPL) (Ginsberg 1998), which is present in adipose tissue and muscle releasing FFA and sn2-monoglycerides for uptake into the adipose tissue (Fredrikson & Belfrage 1983). Conversely, apo C-III is known to inhibit LPL and it may also be involved in inhibiting hepatic uptake of chylomicrons and VLDL remnants (Ginsberg 1998). Apo C-I is only a minor component of the lipoproteins and it may be involved in inhibiting the hepatic uptake of chylomicrons and VLDL remnants (Ginsberg 1998). Hydrolysis of the core triglycerides leads to a reduction in the size of the core volume hence the surface area of the chylomicrons, causing the transfer of phospholipid, free cholesterol; apo C-II and apo C-III back to the HDL (Ginsberg 1998). The remaining chylomicron remnants are then removed from circulation by binding the LDL receptor-related protein (LRP) on the hepatocytes through apoE binding (Beisiegel *et al.* 1989).

1.422 Receptor-mediated endocytosis, uptake by LDL receptors

LDL is the major cholesterol carrying lipoprotein present in the body and transports mainly cholesterol esters. The major apolipoprotein is apoB (Goldstein & Brown 2001). In the periphery triglycerides from the VLDL are removed in muscle and adipose tissue via the actions of LPL leaving VLDL remnants or IDL where the core lipids are cholesterol esters and all other proteins are removed leaving apoB forming a LDL particle (Goldstein & Brown 2001). LDLs can then be cleared from the plasma to the liver via a process known as receptor-mediated endocytosis (Goldstein & Brown 1974), (Figure 1.10 reproduced from Nelson & Cox 2000).

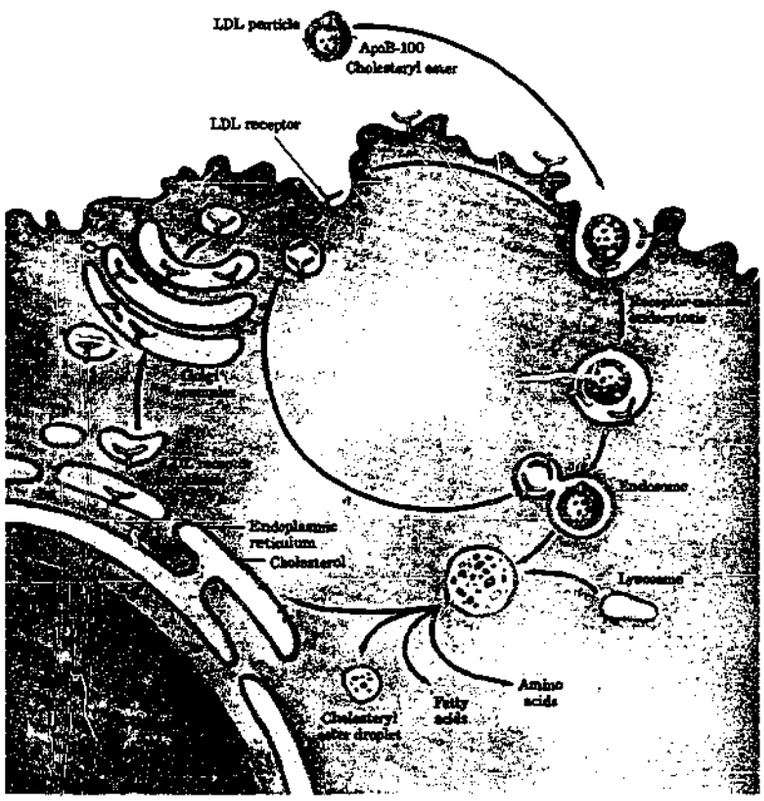


Figure 1.10 Model of receptor mediated endocytosis.
The LDLR is synthesised in the endoplasmic reticulum and moves to the cell surface for ligand binding to occur. The LDL binds to the LDLR and this complex is endocytosed into the cell where it forms an endosome. The receptor is recycled back to the cell membrane and the endosome containing the LDL particle fuses with a lysosome where the LDL is degraded, (Reproduced from Nelson and Cox, 2000)

Receptor mediate endocytosis depends upon cell surface binding, internalisation and intracellular degradation of plasma LDL (Goldstein & Brown 1974). Due to the apoB present on the surface of the LDL it is able to bind to a specific receptor termed the LDL receptor (LDLR). The LDL receptor, first cloned 1983 (Russell *et al.* 1983) is a cell surface glycoprotein containing two asparagine-linked (*N*-linked) oligosaccharide chains and there are approximately 18 serine/threonine-linked (*O*-linked) oligosaccharide chains (Schneider *et al.* 1982; Cummings *et al.* 1983). The LDLR is synthesised in the membranes of the endoplasmic reticulum, then the LDLR make their way through the Golgi complex to the surface of the cell where coated pits are present. Here the LDL can bind to the LDLR, once this occurs the complex is engulfed by the clathrin coated pits and enters the cell (Goldstein *et al.* 1979; Pastan & Willingham 1981; Bretscher & Pearse 1984). This can also occur in the absence of ligand (Basu *et al.* 1981; Anderson *et al.* 1982). Once the complex is inside the clathrin coated pits, they invaginate into the cell, pinching off form clathrin coated endocytic vesicles. The vesicles then shed their clathrin coats, fusing with other vesicles to form endosomes. A drop in the pH causes the LDL to dissociate from the LDLR (Pastan & Willingham 1981; Tycko & Maxfield 1982). The LDLR is recycled and the LDL is then carried further to the lysosomes, where the lysosomal enzymes hydrolyse the apo and the cholesteryl esters of the LDL, resulting in free cholesterol and amino acids. This cholesterol than exerts feedback effects, where it can stimulate ACAT2 allowing cholesteryl ester formation, it can also cause negative feedback by reducing *de novo* cholesterol synthesis, through inhibiting HMG CoA reductase and reducing the rate of LDLR synthesis, via SREBP regulated pathways (described in Section 1.43). The lifespan of the receptor is about 10-30 hours and in this time it participates in this process 150 times without losing function (Goldstein *et al.* 1979; Brown *et al.* 1982).

1.423 Reverse cholesterol transport

The process of reverse cholesterol transport is to transfer peripheral cholesterol to the liver and other steroidogenic tissues. HDL is the lipoprotein which is involved in this process and uptake into the liver occurs through HDL binding to its receptor, SR-B1. SR-B1 is a glycoprotein of 509 residues that contains a large extracellular loop, which is anchored to the plasma membrane adjacent to the short N-terminal and C-terminal domains. (Krieger M. 1999). HDLs are large buoyant particles which are made up of esterified cholesterol. HDLs can be taken up into the liver or other steroidogenic tissue, via SR-B1. SR-B1 mediated binding occurs through binding of the apoB interacting with the receptor (Acton *et al.* 1994; Calvo *et al.* 1997). Unlike LDL uptake via receptor mediated endocyctosis, SR-B1 uptake does not involve lysosomal degradation of the lipoprotein particle, so lipid depleted particles can be released from the cells and begin the cholesterol efflux process again (Krieger M. 1999).

1.43 Regulation of cholesterol synthesis and uptake, Sterol regulatory element binding proteins (SREBP)

Homeostatic regulation of cholesterol is initiated with its conversion to oxysterols, which then signal to molecules to modulate the activity of various transcription factors. The first pathway, which is the focus of this section, is how the cell regulates the amount of cholesterol biosynthesis and uptake via sterol regulatory element binding proteins (SREBP). These are membrane bound transcription factors that are localised on the nuclear envelope and the endoplasmic reticulum (Brown & Goldstein 1997). Target genes of the SREBPs have a sterol response element (SRE), which are present in many genes involved in *de novo* synthesis of cholesterol, including HMG CoA reductase, HMG CoA synthase, farnesyl diphosphate

synthase and squalene synthase. The response element is also on the LDLR gene, which is involved in receptor-mediated uptake of cholesterol (Schoonjans *et al.* 2000). In addition to regulating cholesterol metabolism, these transcription factors have been showed to be involved in lipogenesis, by regulating FAS, ACC, glycerol-3-phosphate acyltransferase and LPL (Schoonjans *et al.* 2000).

There are three known SREBPs designated 1a, 1c and 2, which were first identified in 1993 (Hua *et al.* 1993; Yokoyama *et al.* 1993; Briggs *et al.* 1993; Wang *et al.* 1993). SREBP 1a and 1c are produced from alternate splicing of a single gene in humans, hamsters and mice (Yokoyama *et al.* 1993; Hua *et al.* 1995; Shimomura *et al.* 1997). A separate gene encodes the third SREBP, 2, and to date only one transcript has been found in humans, hamsters and mice (Hua *et al.* 1993; Miserez *et al.* 1997). All three have a similar tripartite structure, namely there is an NH_2 terminal transcription factor domain; a middle hydrophobic region containing two hydrophobic transmembrane segments and a COOH-terminal regulatory domain. Next to the NH_2-terminal is a basic-helix-loop-helix-leucine zipper domain and at the COOH-terminal end is a regulatory domain. The NH_2-terminal and the COOH-terminal are facing the cytoplasm with the luminal loop projecting into the lumen of the organelle (Hua *et al.* 1995; Duncan *et al.* 1997).

SREBP is associated with another protein, SREBP cleavage activating protein (SCAP), which acts to regulate sterol sensing (Schoonjans *et al.* 2000). The SCAP protein is made up of two principal domains, an NH_2-terminal domain, which like polytopic membrane proteins contains alternating hydrophobic and hydrophilic segments (Hua *et al.* 1996; Nohturfft *et al.* 1998) and the COOH-terminal, which has five 'WD' repeats, which are known to regulate protein-protein interactions (Sakai *et al.* 1997). In the case of SCAP and SREBP interaction it

has been shown that it is these 'WD' domains in COOH-terminal that specifically interact with the COOH-terminal of SREBP (Sakai *et al.* 1997).

Sterol depletion in cells leads to the initiation of a proteolytic cascade (Figure 1.11, reproduced from Brown & Goldstein 1999), where SCAP escorts SREBP from the endoplasmic reticulum to Golgi compartment where site 1 protease (S1P) cleaves SREBP at site 1, which is located in the middle of the luminal loop, between leucine and serine (Duncan *et al.* 1997). This cleavage at site 1 breaks the covalent bond between the transmembrane domains, however they are still attached to the membranes and thus allow the second protease, site 2 protease (S2P), to cleave at a Leu-Cys bond at the NH2-terminal at site 2. This releases the NH2-terminus into the nucleus, where it is able to activate transcription of the HMG CoA reductase, HMG CoA synthase and the LDLR genes and increase cholesterol production and uptake (Brown & Goldstein 1997; Brown & Goldstein 1999). SCAP is also a sterol sensor, and when sterols bind to SCAP it inhibits site 1 cleavage, thereby reducing cholesterol production and uptake (Brown & Goldstein 1997).

1.44 Bile acid pathway

The elimination of cholesterol is through the synthesis of bile acids. Bile acids are secreted into the small intestine where they are essential in the solubilisation and absorption of dietary cholesterol as well as fat-soluble vitamins. In the classical pathway of bile acid production cholesterol 7α-hydroxylase is the rate-limiting step (Russell & Setchell 1992), which is encoded by the *Cyp7a* gene. Null mice for this gene have been generated and they die three weeks after birth unless supplied with dietary fat-soluble vitamins and cholic acid (Ishibashi *et al.* 1996; Schwarz *et al.* 1996), highlighting the importance of bile salts in the intestinal absorption of fat soluble molecules. Regulation of this pathway occurs in both a feed forward and feed back manner, by regulating *Cyp7a* and thus ultimately cholesterol 7α-hydroxylase

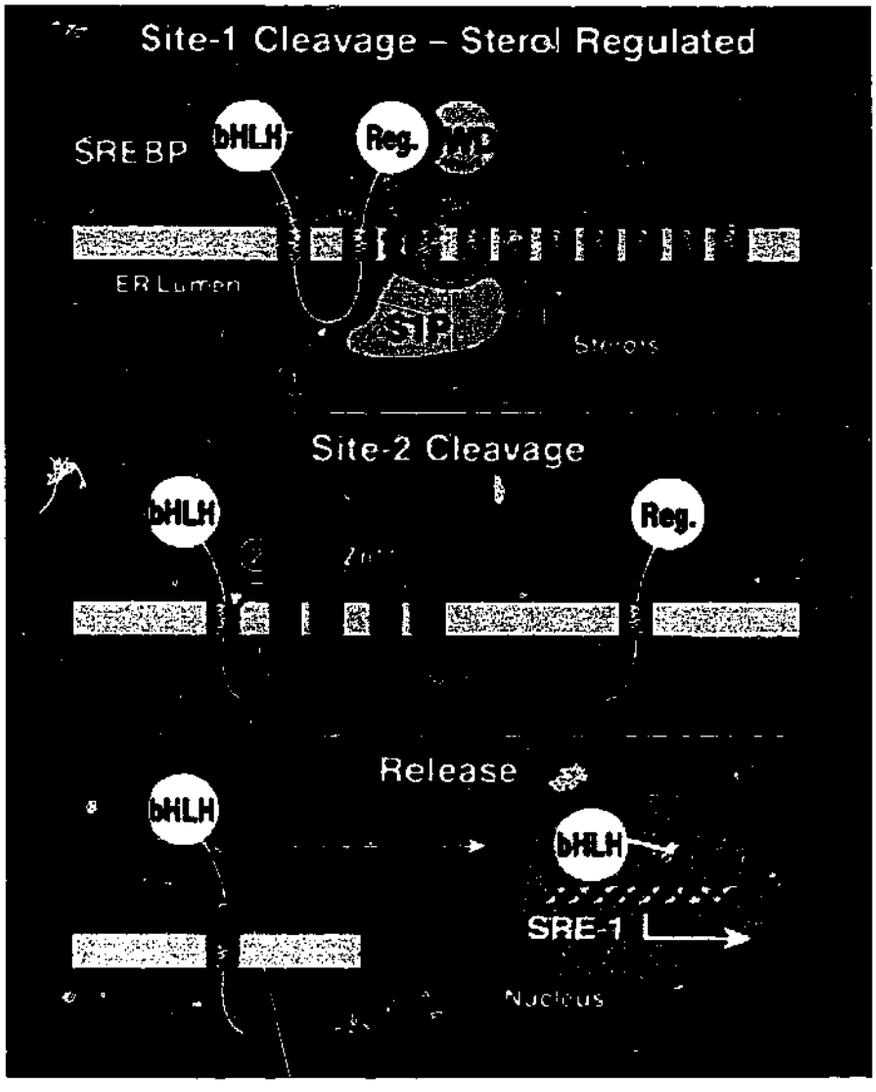


Figure 1.11 Model of sterol regulation of SREBP cleavage.
Top panel, represents absence of sterol leading to site 1 cleavage by S1P. SCAP regulates this cleavage. Middle panel, site 2 cleavage occurs by S2P once SREBP is in two halves. This cleavage occurs at the NH2-terminal bHLH-Zip domain of SREBP. Bottom panel, the second cleavage allows to the bHLH-Zip domain to leave the membrane, with three of the hydrophobic residues present in the COOH-terminus. This mgrates into the nucleus, binding to the SRE of target genes initiating transcription (Reproduced from Brown and Goldstein 1999).

(Figure 1.12). Elevations in hepatic cholesterol lead to the formation of oxysterols that activate liver X receptor α (LXRα), which heterodimerises with 9-cis retinoic acid receptor (RXR). This can then bind to a LXR-RE in the *Cyp7a* promoter (Janowski *et al.* 1996; Forman *et al.* 1997b) initiating transcription of mouse *Cyp7a*; hence increasing bile acid production. Mice lacking LXRα are unable to induce transcription of *Cyp7a* in response to cholesterol feeding (Peet *et al.* 1998), eloquently demonstrating the importance of LXRα in stimulating *Cyp7a* expression. In addition to LXRα binding to the *Cyp7a* promoter region, another nuclear orphan receptor liver receptor homolog-1 (LRH-1), also known as CPF and FTF, has been shown to have a binding site present on *Cyp7a* only 70bp from the LXRα site (Russell 1999). Bile acid formation activates farnesoid X receptor (FXR), a bile acid receptor (Wang *et al.* 1999; Makishima *et al.* 1999; Parks *et al.* 1999). As there is no FXR-binding sites on the *Cyp7a* promoter (Castillo-Olivares & Gil 2000), its inhibition is via indirect mechanisms by inducing the transcription of short heterodimer partner (SHP). This can form a heterodimeric complex with LRH-1, and this binding causes an inhibition of *Cyp7a* synthesis, thereby reducing bile acid synthesis (Lu *et al.* 2000; Goodwin *et al.* 2000).

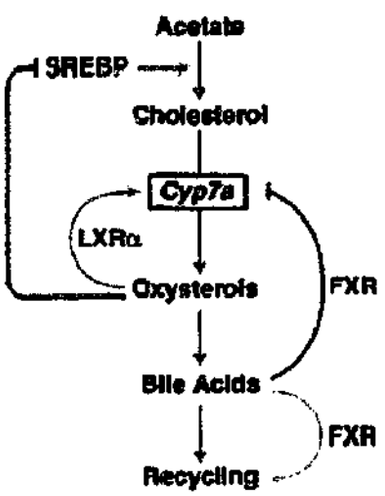


Figure 1.12: Model of bile acid regulation.

Cyp7a encodes cholesterol 7α-hydroxylase, which is the rate-limiting step in the formation of bile acids. It is positively regulated by LXRα and negatively regulated by FXR (Russell 1999).

The alternative pathway in bile acid production is activated by cholesterol conversion to oxysterol

that contain a hydroxyl group at either carbon position 24, 25, 27 on the side chain. These intermediates are substrates for an oxysterol 7α-hydroxylase, which is encoded by the *Cyp7b* gene, thereby producing 7α-hydroxylated oxysterols which are then able to be funnelled into the classical pathway of bile acid production (Schwarz *et al.* 1998).

In addition to the cholesterol 7α-hydroxylase mediation of bile acids, other mechanisms are are also responsible for removing cholesterol. Recently identified ABCG transporters G5 (Lee *et al.* 2001) and G8 have also been implicated in this transport (Berge *et al.* 2000). ABCG transporters are integral membrane proteins that transport mainly plant sterols but also cholesterol across cellular membranes via energy derived from the hydrolysis of ATP. These transporters form heterodimeric complexes that allow the transport of sterol and some cholesterol, from the small intestine and from the liver, for excretion through the bile acid pathway. Like *Cyp7a*, these transporters are also regulated by the transcription factor LXR (Berge *et al.* 2000). It is via these pathways that cholesterol clearance from the body is regulated.

1.45 Regulation of cholesterol homeostasis by estrogen

As previously described, when women progress from a pre- to post-menopausal state there is a dramatic reduction in circulating estrogens, coinciding with an elevation in serum LDL and a reduction in serum HDL (described in Section 1.1). This can be reversed with E_2 replacement. Additionally, men with reported aromatase deficiencies and a non-functioning ER also have disrupted cholesterol homeostasis, (described in Section 1.23), indicating an important role for estrogen in regulating pathways involved in cholesterol homeostasis.

Therefore estrogen's regulation of these pathways has been studied by a number of investigators.

1.451 Estrogen action on HMG CoA reductase regulation

The promoter of HMG CoA reductase has been shown to contain an estrogen-responsive element-like sequence, termed Red-ERE (Di Croce *et al.* 1999), but whether estrogen acts on this sequence *in vivo* is unclear. To date studies examining the relationship between estrogen and HMG CoA reductase have provided variable results. In MCF-7 cells estrogen was able to induce HMG CoA reductase, which was dependent on the ERE and this stimulation could be inhibited by the anti-estrogen, ICI 164,384, but this did not occur in hepatic cell lines (Di Croce *et al.* 1999). It appears as if estrogen actions on HMG CoA reductase in hepatic cell lines and *in vivo* (rat livers) are at the level of protein rather than mRNA (Di Croce *et al.* 1996; Di Croce *et al.* 1999). When intact female rats were treated with physiological levels of E_2, HMG CoA reductase activity was stimulated (Parini *et al.* 2000). Conversely, in males pharmacological levels of E_2 (10 mg/kg of E_2) caused a reduction in HMG CoA reductase activity (Marino *et al.* 2001). This research shows variable results in HMG CoA reductase regulation by estrogen and that possibly any regulation may be posttranscriptional. Further research needs to be performed to fully understand its regulation.

1.352 Estrogen actions on the LDLR

In addition to estrogen having a regulatory effect on HMG CoA reductase, it has also been shown to stimulate LDLR expression (Di Croce *et al.* 1996; Parini *et al.* 2000). E_2 treatment has been shown to cause an increase in both the mRNA and protein expression of the LDLR. These effects have been seen in both male and female rats. One study showed that in male rats receiving a dose of 10mg/kg of E_2, after 30 min there was a rapid increase in LDLR

mRNA levels and after 1 hr increases in protein expression of the LDLR could be seen. The authors also showed that this coincided with induction of the intracellular signal transduction pathway leading to IP_3 and PKC–α translocation to the membrane, and that this may possibly be the mechanism for hormonal stimulation of the LDLR (Marino *et al.* 2001). Estrogen stimulation of the LDLR has also been shown in intact female rats, although this occurred only at high doses of E_2 treatment, (1mg/kg and 4 mg/kg). No stimulation occurred at the lower doses (Parini *et al.* 2000), indicating that only the pharmacological doses affect the LDLR. This stimulation of the LDLR by E_2 treatment has been shown to be reliant on the ER as treatment with tamoxifen or clomiphene, known ER antagonists, were able to inhibit this stimulation (Parini *et al.* 2000). However, treatment with these antagonists alone had no effect on the LDLR (Parini *et al.* 2000). This ER mediated stimulation of the LDLR has been shown to be a specific interaction of the ERα with the transcription factor Sp1 bound to the promoter of the LDLR (Li *et al.* 2001). Additionally, growth hormone (GH) may also play a role in E2 stimulation of the LDLR, as when hypophysectomized rats were treated with E_2 there was only a slight increase in the LDLR mRNA levels, but GH replacement was able to restore the E_2 induction of the LDLR (Rudling *et al.* 1992). These studies clearly show a role for estrogen in regulating the LDLR and that it may possibly be a regulating factor in elevated serum LDL levels in postmenopausal women.

1.353 Estrogen actions on Cholesterol 7α-hydroxylase

As mentioned earlier, *Cyp7a* encodes cholesterol 7α-hydroxylase, which is the rate-limiting step in the production of bile acids (described in Section 1.44). Estrogen treatment has been shown to have varied effects on the *Cyp7a* gene and the activity of the enzyme. One study showed that rats treated with 17β–estradiol-3-benzate at 25μg/day for 20 days, induced a

three-fold increase in the activity of cholesterol 7α-hydroxylase after two different feeding conditions. One was after fasting and being killed in the morning and the other involved non-fasted animals, killed at night (Ferreri & Naito 1977). Another study also performed in rats showed an increase in the activity of cholesterol 7α-hydroxylase after estradiol treatment (Deliconstantinos & Ramantanis 1982). *In vitro* studies have also revealed a role for estrogens in the regulation of cholesterol 7α-hydroxylase. In this case, liver microsomes were isolated from rats which had been fed either a standard diet, where they were fed ad libitum or fasted for 24 hours, or were fed a diet containing the bile acid sequesterant cholestyramine. Administration of 5μM estradiol stimulated the activity of cholesterol 7α-hydroxylase in the rats that had either been fasted or fed the cholestyramine diet (Chico *et al.* 1994). Transient increases in cholesterol 7α-hydroxylase activity have also been shown in hepatocytes cultured from rats (Chico *et al.* 1996). In addition to studies in rats, regulation of cholesterol 7α-hydroxylase by estrogens has also been studied in nonhuman primates. One study used female cynomolgus monkeys that were ovariectomised and fed a moderately atherogenic diet for 12 weeks in addition to estrogen administration. Estrogen treatment led to significant reduction in hepatic cholesterol levels and mRNA levels of *Cyp7a* were elevated (Colvin, Jr. *et al.* 1998). A similar study was performed in baboons and it also showed an increase in cholesterol 7α-hydroxylase activity (Kushwaha & Born 1991). Whilst many studies have shown that administration of estrogen leads to stimulation of cholesterol 7α-hydroxylase the exact mechanisms for this action are yet to be elucidated.

1.454 Estrogen actions on SR-B1

Estrogen has been shown to reduce hepatic uptake of HDLs in rats (Rinninger & Pittman 1987) and subsequently this reduction may be a direct consequence of estrogen's ability to

suppress hepatic SR-B1 expression (Landschulz *et al.* 1996). Specifically, it is SR-B1 expression on the parenchymal cells (hepatocytes) which is reduced (Fluiter *et al.* 1998; Fluiter *et al.* 1999), whereas estrogen treatment can increase SR-B1 expression in hepatic Kupffer cells (sinusoidal macrophages), which are far less abundant (Fluiter *et al.* 1998). Again estrogen has the ability to influence cholesterol uptake and this may also be playing an important role in regulating serum cholesterol levels.

1.5 Aims

Although, epidemiological evidence suggests that estrogen plays an important role in lipid homeostasis specifically studies examining the cholesterol pathway show variable results, whilst studies on the triglyceride pathway are unclear on the mechanisms behind estrogen's contribution. Therefore this thesis aims to elucidate the complete role of estrogen in lipid homeostasis using the estrogen deficient ArKO mouse.

The first aim, of this thesis is to determine the role of estrogen in triglyceride homeostasis using the estrogen deficient mouse model. In this study male and female mice will be investigated, examining the morphology of their liver, and measuring serum and hepatic triglyceride levels. To understand the mechanisms leading to the disruptions in these pathways, real time PCR will be used to examine gene expression of key enzymes and proteins.

The second aim of this project is to examine the consequences of estrogen replacement on lipid metabolism, and in particular, triglyceride turnover in the liver.

The third aim of this thesis is to define the role of estrogen in regulating cholesterol homeostasis by challenging the male and female ArKO mice with high cholesterol diets. Included in this series of experiments will be an investigation of how this dietary intervention

affects cholesterol metabolic pathways in estrogen deficient mice, by examining hepatic and serum cholesterol levels in the presence or absence of estrogen and using real time PCR to measure gene expression of key enzymes in these pathways.

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Chapter Two: Estrogen Deficiency Leads to a Sexually Dimorphic Phenotype of Hepatic Steatosis in Male Mice

Submitted to Endocrinology, In Revision.

Declaration for Thesis Chapter Two

In the case of Chapter two, contributions to the work involved the following:

Name	Percentage contribution	Nature of contribution
Kylie Hewitt	89%	All experimental work Preparation of manuscript
Wah Chin Boon	2%	Experimental assistance with Light cycler Editing of manuscript
Yoko Murata	2%	Experimental assistance with Light cycler and triglyceride assay
Margaret Jones	2%	Supervisor, Assistance with triglyceride assay assistance with planning of manuscript
Evan Simpson	5%	Supervisor intellectual input Editing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
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Estrogen deficiency leads to a sexually dimorphic phenotype of hepatic steatosis in male mice.

Regulation of triglycerides in ArKO mice

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Abstract

The aromatase knockout (ArKO) mouse cannot synthesize endogenous estrogens due to disruption of the *Cyp*19 gene. Previously, we have shown that the ArKO mice have an age progressive obese phenotype in both genders, and a sexually dimorphic alteration to hepatic cholesterol homeostasis. The present study examined the livers of 24-26 wk old ArKO mice for disruption to triglyceride and fatty acid homeostasis, before and after being challenged for 90 d on a high cholesterol diet. Estrogen deficiency in males significantly increased hepatic triglyceride levels, causing hepatic steatosis, which was reversed by cholesterol feeding. Transcripts of enzymes and proteins encoding genes regulating *de novo* synthesis, fatty acid uptake and VLDL secretion were measured. Estrogen deficiency in males lead to a significant increase in expression of both acetyl CoA carboxylase α and fatty acid synthase, indicative of increased *de novo* fatty acid synthesis, however cholesterol feeding had no effect. Adipocyte differentiated regulatory protein (ADRP) was also increased in estrogen deficient males, indicating an increased fatty acid uptake. Cholesterol feeding significantly increased the expression of ADRP in both genotypes; however CD36 expression was decreased by cholesterol feeding in the ArKO males. Despite increased hepatic triglycerides in ArKO males, apoE and microsomal triglyceride transfer protein expression were not changed, suggesting no compensatory increase in VLDL secretion. Conversely, ArKO females presented with no hepatic phenotype despite their obesity, although they had elevated serum triglyceride levels. This study reveals estrogen deficiency leads to a severe impairment in hepatic triglyceride homeostasis in males but not females, resulting in hepatic steatosis.

Introduction

Estrogen is known to promote the accumulation of gluteo-femoral fat (1,2). After menopause, when plasma estrogen levels decline dramatically, there is a switch in adipocyte accumulation from the gluteo-femoral region to the intra-abdominal area, which is associated with development of a metabolic syndrome (1,3,4). The 'Metabolic Syndrome' has been linked with an increased risk of cardiovascular disease and features of this syndrome include central obesity, elevated triglyceride levels, increased low-density lipoproteins (LDL) and decreased high density lipoproteins (HDL) (1,5). Central obesity is closely associated with insulin resistance and a consequence of insulin resistance is elevated free fatty acids, which may impact on hepatic function (1,6). In menopausal women, triglyceride levels are elevated compared with premenopausal women and are highly correlated with increased abdominal fat (7). Men are also known to have higher levels of triglycerides compared with women (1). This evidence associates estrogen with obesity as well as triglyceride homeostasis.

Mouse models devoid of estrogen and lacking estrogen action have been generated, and they too have disrupted lipid homeostasis. The aromatase knockout (ArKO) mouse presents with age-progressive obesity and hepatic steatosis, and by one year of age develop hypercholesterolemia, hyperinsulinemia, hyperleptinemia and the male ArKO mice have hypertriglyceridemia (8). Estrogen receptor (ER) α and the ERαβ double knockout mice present with a similar obese phenotype to the ArKO (9,10), although the ERβ knockout mice have no lipid phenotype (10). None of the three ER null models have been reported to present with hepatic steatosis or any other liver phenotype. A molecular characterisation of the ArKO obese phenotype revealed an increase in lipoprotein lipase (LPL) in the adipose tissue, which may account for the

increase in adiposity (11), again suggesting a role for estrogen in regulating triglyceride homeostasis.

Previously, we have shown that the ArKO mice have altered hepatic cholesterol homeostasis in both male and female ArKO mice (12). Although there is sexual dimorphism in this presentation, it is not the cause of the hepatic steatosis in the male ArKO mice (12). Toda *et al* (13) generated another ArKO mouse also by disrupting exon 9 of the *Cyp19* gene. They also found the presence of hepatic steatosis as well as a disruption of β-oxidation at the level of gene expression and the catalytic activity of the β-oxidative enzymes. This was reversed by 17β-estradiol replacement (14).

In addition to mouse models, men with mutations in aromatase (15,16) and the ERα (17) have been reported. These patients also present with disruption to lipid homeostasis and one of the aromatase deficient men has been reported to have hepatic steatosis (18).

Triglyceride accumulation within the liver results from a loss balance between the rate of fatty acid synthesis and oxidation, the rate of VLDL secretion and the rate of free fatty acid uptake up from the serum (19). These are derived primarily as a consequence of lipolysis in the adipose tissue, which is elevated in type 2 diabetes (19).

In this present study, we show that the ArKO mice develop a sexually dimorphic phenotype of hepatic steatosis, namely, the condition only manifests in the males. This appears to be due, in part, to differential expression of key enzymes and factors involved in triglyceride metabolism. Moreover, the accumulation of triglycerides is prevented by cholesterol feeding.

Methods

Mice

The ArKO mice were generated by deleting 90% of exon IX of the *Cyp19* gene as described in Fisher *et al* (20). WT and homozygous null offspring were generated by heterozygous matings. The genotype of the offspring was determined by PCR as described by Robertson *et al* (21). The animals were housed in specific pathogen free conditions and had unlimited access to drinking water and food.

Diets

Soy free mouse chow (Glen Forest stock feeders) is the control diet used to feed the mice; it does not contain soy products, which are found in regular mouse chow, as isoflavones in soy are known to have estrogenic effects (22). This diet contains 15% of calories as fat (0.02% cholesterol), 20% calories as protein and 65% of calories as carbohydrate. Intermediate and high cholesterol diets were fed to the mice to challenge their lipid homeostasis. The intermediate diet had 0.2% cholesterol added to the soy free mouse chow; this was 2 fold higher than normally needed to maintain homeostasis. The cholesterol high diet had 2% cholesterol added to the soy free mouse chow, which was 20-30 fold higher than that normally needed to maintain homeostasis. ArKO and WT males and females were fed control diet (0% added cholesterol to a soy free diet), or the 0.2% cholesterol diet or the 2% cholesterol diet for 90 days beginning at 10-12 weeks of age. A food intake study was performed where 10g of food per day per animal were given to the mice over a 5-day period. Food intake was calculated as the starting amount of food of 10g minus the amount of food remaining each day.

Tissue Collection and Histology

Mice were weighed then killed by cervical dislocation. Truncal blood was collected after decapitation. Blood was allowed to clot, serum collected and stored at -20 °C. The liver was removed, weighed and snap frozen in liquid nitrogen and stored at -80°C for gene and lipid analysis. Part of the liver was immersion fixed in Bouins fixative then stored in 70 % ethanol. Fixed samples were embedded in a random orientation in paraffin and sliced into 7 µm sections. Sections were then stained with hematoxylin (Sigma, USA), counterstained with eosin (H&E) and coverslipped with DPX (BDH, England).

Measurement of serum and hepatic lipids

Hepatic triglycerides were measured following extraction with chloroform/methanol (2:1v/v) (23). Briefly, 0.2 g of liver was homogenized in 10ml of chloroform/methanol. Samples were centrifuged for 20 min at 800 g, the lipid phase was removed and chloroform was evaporated. The triglycerides were then measured using Triglyceride Kit (320-A, Sigma, USA). Triglycerides from the serum were quantified using triglyceride flex (Dade Behring, Newark, DE, USA).

Measurement of fatty acid β–oxidation

Fatty acid β-oxidation was measured as described by Nemoto *et al* (14). Briefly, 250mg of fresh liver were homogenised in four volumes of 0.25M sucrose containing 1mM EDTA. Ten µl of homogenate was incubated in assay buffer of as described (14) with [1-14C] palmitic acid (C16:0) (Amersham Pharmacia Biotach, UK). The reaction was conducted for 25 min at 25oC. The radioactive degradation products in the water phase were counted on a 2500TR liquid scintillation analyser (Packard, ACT, Australia).

Gene Analysis

RNA was extracted from the liver using the phenol-chloroform method (Ultraspec RNA, Fisher Biotech, Australia) and quantified spectrophotometrically. Two-step RT-PCR was performed using random primers (Roche, Germany) and AMV reverse transcriptase enzyme (Promega Life Sciences, USA). Lightcycler™ (Roche, Germany) was used to quantitate mouse transcripts using specific primer pairs (Table 1). Primer pairs were shown to be specific through single peak melting curves and a single product was detected on an ethidium bromide (Sigma, USA) 1% agarose (Promega, Life Sciences, USA) gel in 1 X TBE corresponding to the appropriate product size as measured by a 1kb ladder (Promega, Life Sciences, USA).

All samples were normalised to 18S transcript levels. All samples were run individually in three separate reverse transcription reactions and transcripts measured using real time PCR. Then the data presented is the mean of three consistent runs. Inter-assay variability was assessed using a quality control of RNA that was reverse transcribed and used in each individual run.

Statistical Analysis

All graphs are expressed as mean ±SEM. Univariant two way ANOVA was used to determine overall statistical differences. Genotypes within a diet were compared using univariate ANOVA. In the experiments involving three diets, Tukey's post hoc test was used to determine significance (SPSS version 10.0 for Windows, SPSS, Inc, Chicago, IL, USA). A minimum of five animals were used in each group. Numbers are indicated in the figures.

Table 1. Primer sequences and product size

Gene	Primer pairs	Product size(bp)
ACCα	F: 5'-TGTTTGGGGTTATTTCAGTGTTGC-3' R: 5'-TGTCCAGCCAGCCAGTGTCG-3'	236
ACCβ	F:5'-CCGTGCCCTGTGCCAACCATA-3' R:5'-GCAGCCGCTCCCCTTCATTCT-3'	171
ADRP	F:5'-ACCTTGTGTCCTCCGCTTATGTCA-3' R:5'-GTTACGGCACCTCTGGCACTGG-3'	259
Apo E	F:5'-CGAGGGCGGCTGGAGGAAGTG-3' R:5'-TGGGGTGATGATGGGGTTGGTAGC-3'	243
CD36	F:5'-TGTTCTTTGGCTTGGTTTTCACG-3' R:5'-TGTCACCCTGCTCATTTCCCTCTG-3'	268
FAS	F:5'-CACAGATGATGACAGGAGATGGA-3' R:5'-TCGGAGTGAGGCTGGGTTGATA-3'	205
FATP2	F:5'-CCCTTCCTGCTGTTCCGAGACGAG-3' R:5'-CCCCGCAGCATTGAAAGCAGTG-3'	257
FATP5	F:5'-CTCCCTGCCTATGCCACACCTCA-3' R:5'-ATCCCCACATCAAAACCCTCACG-3'	113
MTTP	F:5'-AGAGGCTGGGCTGGAGTT-3' R:5'-TCTGGCTGAGGTGGGAATAC-3'	240
SR-B1	F:5'-ACGCCGACCCTGTGTTGT-3' R:5'-CCTGTTTGCCCGATGCCCTTGAC-3'	166
18S	F:5'-CGGCTACCACATCCAAGGAA-3' R:5'-GCTGGAATTACCGCGGCT-3'	180

Results

Body Weight

ArKO males and females on the control diet were significantly heavier compared to diet matched wildtype (WT) mice (p=0.026, p=0.019, respectively; Fig1A and B). Feeding of the 2% cholesterol diet resulted in a significant reduction in the body weight of ArKO mice compared with ArKO mice on the control diet, for both males and females (p=0.002, p=0.037, respectively; Fig 1A and B).

To ensure that the weight lost was a physiological effect and not an aversion to the food, a food intake study was preformed. There was no difference in food intake between any of the three diets (data not shown).

Liver Weight

ArKO male mice on the control diet had significantly elevated liver weight compared to WT controls (p=0.013, Fig 2A). The cholesterol diet also had an effect on liver weight, where the 0.2% and 2% cholesterol diets led to a significant reduction in liver weight in ArKO mice compared to ArKO mice on the regular diet (p=0.046, Fig 2A). Females on the other hand, displayed no differences in liver weight between ArKO and WT on the control diet (Fig 2B). The 2% cholesterol diet also had no effect on the liver weight of either the ArKO or WT females (Fig 2B).

Liver Morphology

Gross morphology of livers of the ArKO male mice revealed pale and enlarged livers Closer examination of liver morphology was performed using paraffin sections stained with haematoxylin and eosin (H&E). Examination of the liver sections revealed that the increased liver weight seen in the control fed ArKO male mice is most likely due to the large accumulation of lipids in their livers, which was absent from WT controls

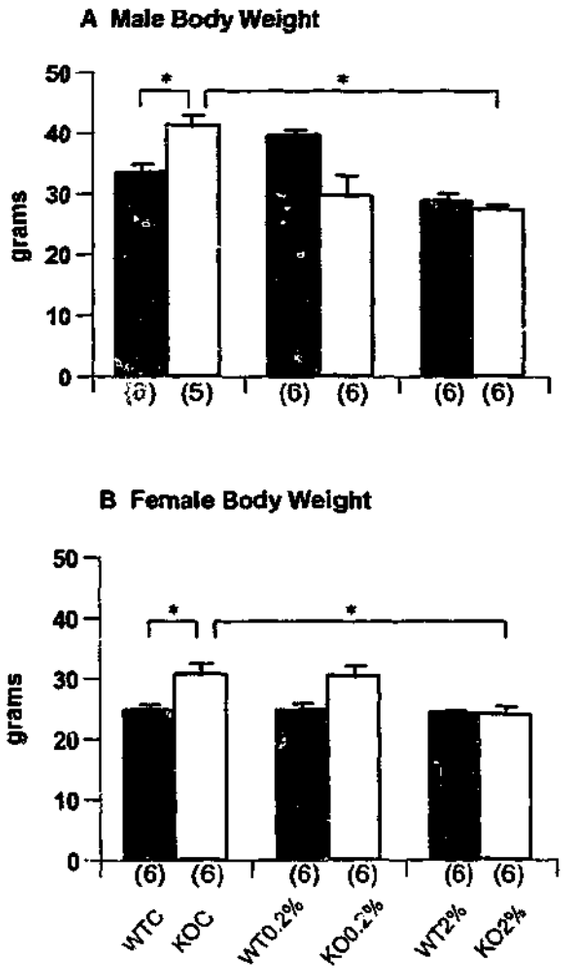


Figure 1. Body Weight.
A Male body weight. ArKO control mice were significantly heavier compare with WT control mice, *p<0.05. ArKO mice on the 2% cholesterol diet had significantly reduced body weight compared with ArKO control mice, *p<0.05. WT mice no changes. **B Female body weight.** ArKO control mice were significantly heavier compared WT control mice, *p<0.05. Cholesterol feeding significantly reduced their body weight, *p<0.05. WT mice no changes.
ArKO (KO) mice white bar, WT mice black bar. C refers to control diet, 2% refers to 2 % cholesterol diet.

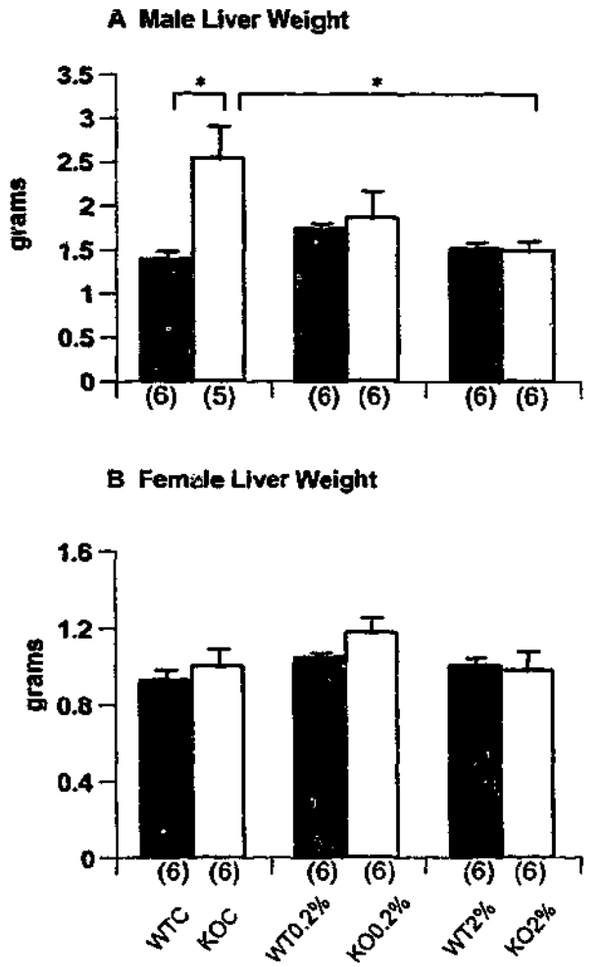


Figure 2. Liver Weight.

A Male liver weight. ArKO control mice had significantly elevated liver weight compared with WT controls, *p<0.05. Cholesterol feeding led to a significant decrease in liver weight, *p<0.05. WT mice no changes. **B Female liver weight.** Genotype and diet had no effect on liver weight.

ArKO (KO) mice white bar, WT mice black bar. C refers to control diet, 2% refers to 2 % cholesterol diet.

(Fig 3A and D). This phenotype was reduced when male ArKO mice were fed the 0.2% cholesterol diet, as there was a reduction in the lipid accumulation of the livers of the ArKO mice (Fig 3B and C). This appears to be dose dependent as the livers of ArKO male mice on the 2% cholesterol diet appeared completely normal (Fig 3C). WT males on all three diets did not display any hepatic steatosis (Figs 3D, E and F). In accordance with the above liver weight data, the female mice showed no difference in liver morphology, regardless of genotypes or diets (Fig 3G, H, I, J, K and L).

Hepatic triglyceride levels

Hepatic and serum triglyceride levels were measured in both male and females. Significantly elevated levels of triglycerides were present in the livers of the ArKO male mice compared to WT controls ($p=0.001$, Fig 4A). This may account for the lipid accumulation observed in the ArKO male mice livers. These levels of triglycerides were significantly reduced in the livers of male ArKO mice when they were fed the 0.2% and 2% cholesterol diets, thus possibly explaining the reduction in lipid accumulation observed in figure 3A ($p=0.000$, Fig 4A). In contrast, female mice displayed no differences in hepatic triglyceride levels between ArKO and WT control mice. Female mice of both genotypes on the 0.2% cholesterol diet had a significant increase in hepatic triglyceride levels ($p=0.008$, Fig 4B). This was also increased in female mice on the 2% cholesterol diet, however this did not reach statistical significance ($p=0.065$, Fig 4B).

Serum triglyceride levels

Serum triglyceride levels were also measured in male and female mice. No difference was seen in serum triglyceride levels between male ArKO and WT mice on the control diet (Fig 5A). When the male mice were fed the 2% cholesterol diet there was a significant reduction in serum triglyceride levels in the ArKO male mice compared to

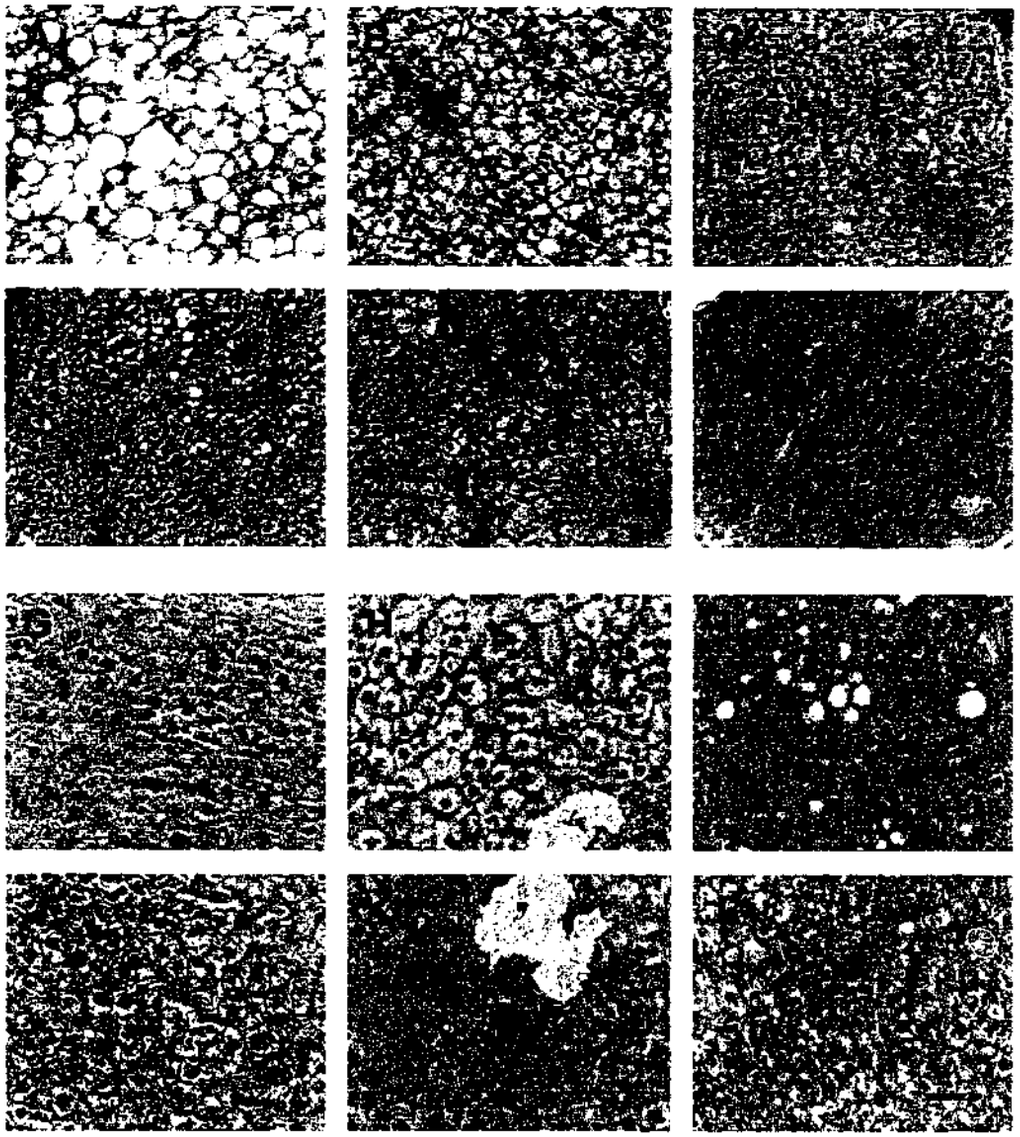


Figure 3. Liver Morphology.
A Male ArKO control, presence of hepatic steatosis. **B** Male ArKO 0.2% cholesterol diet, decreased levels of hepatic steatosis compared with ArKO control. **C** Male ArKO 2% cholesterol diet, normal morphology. **D** Male WT control, normal morphology. **E** Male WT 0.2% cholesterol diet, normal morphology. **F** Male WT 2% cholesterol diet, normal morphology. **G** Female ArKO control, normal morphology. **H** Female ArKO 0.2% cholesterol diet, normal morphology. **I** Female ArKO 2% cholesterol diet, normal morphology. **J** Female WT control, normal morphology. **K** Female WT 0.2% cholesterol diet, normal morphology. **L** Female WT 2% cholesterol diet, normal morphology. Scale Bar 100μm.

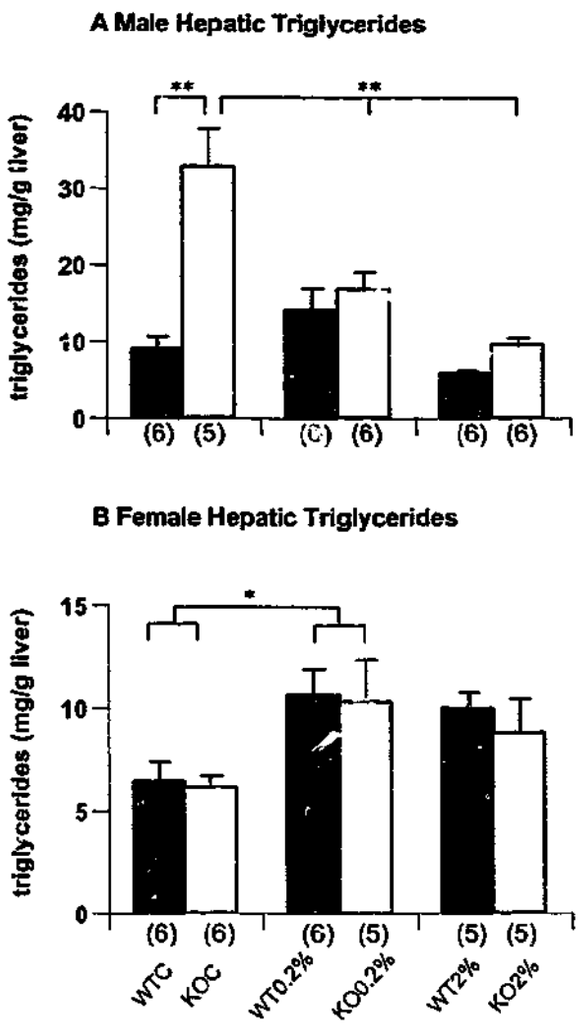


Figure 4. Hepatic Triglyceride Levels.

A Male Triglyceride Levels. ArKO control mice had significantly elevated hepatic triglyceride levels compared with WT control mice, $p<0.01$. Cholesterol feeding significantly reduced these levels, $p<0.01$. WT mice on 0.2% cholesterol diet had significantly elevated levels compared with WT mice on the 2% cholesterol diet, $p<0.05$. **B Female Triglyceride Levels.** Control mice had significantly lower levels of hepatic triglycerides compared with those fed the 0.2% cholesterol diet, $p<0.05$. There were no other differences between groups.

ArKO (KO) white bar, WT black bar. C refers to control diet and 2% refers to 2% cholesterol diet.

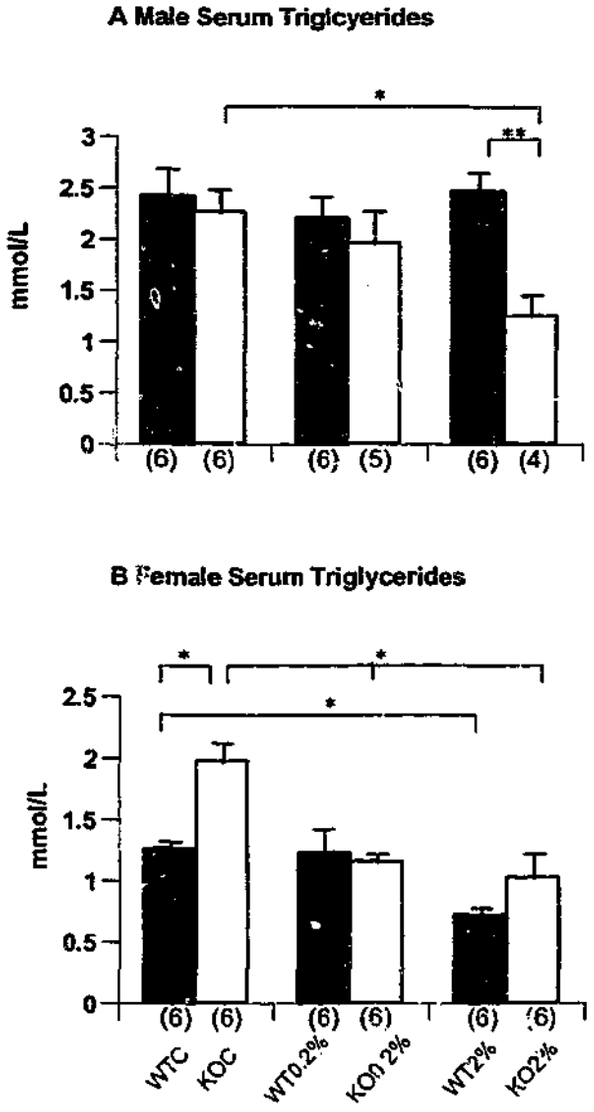


Figure 5. Serum Triglyceride Levels.

A Male Triglyceride Levels. No differences between control mice. Cholesterol feeding significantly reduced these levels, between ArKO control and ArKO 2% cholesterol **p< 0.01 and between WT 2% and ArKO 2%, *p<0.05. **B Female Triglyceride Levels.** ArKO controls had significantly higher levels of serum triglycerides compared with WT controls, p<0.05. Cholesterol feeding significantly reduced serum triglyceride levels between ArKO control mice and ArKO mice fed 2% cholesterol, *p<0.05. And between WT control mice and WT mice fed 2% cholesterol, *p<0.05.

ArKO (KO) white bar, WT black bar. C refers to control diet and 2% refers to 2% cholesterol diet.

diet matched WT mice and ArKO mice on the controls diet (p=0.901, Fig 5A). Conversely, ArKO female mice, had significantly elevated levels of serum triglycerides compared to WT controls (p=0.003, Fig 5B). The 0.2% and 2% cholesterol diets led to a significant reduction in serum triglycerides levels in the female ArKO mice (p=0.005 and p=0.002, respectively Fig 5B). However, the 2% cholesterol diet also significantly reduced the serum triglyceride levels in the WT female mice compared with WT control mice, (p=0.034, Fig 5B).

Fatty acid β- oxidation assay

In order to investigate if a change in the rate of oxidation of fatty acids could be a factor in the triglyceride accumulation in the male ArKO mice. The rate of FA oxidation was measured by ^{14}C-palmitic acid break down. There were no changes in oxidation between WT and ArKO male mice on the control diet. There was a trend for an increase between WT controls and WT mice on the 2% cholesterol diet, (p=0.077), but no difference between ArKO mice. Females also showed no difference between genotypes on the control diet (data not shown). Thus we conclude that changes in fatty acid oxidation do not contribute to the differences in triglyceride accumulation.

Gene expression of enzymes involved in fatty acid synthesis

To gain an understanding of the mechanisms that led to increased hepatic triglycerides and the apparent reversal of this phenotype with the addition of cholesterol to their diets, real-time PCR was performed to quantitate expression of genes involved in fatty acid synthesis, transport and export. Transcripts were measured in ArKO and WT mice on the control and 2% cholesterol diets.

Acetyl CoA carboxylase α (ACCα) is a key regulatory enzyme in *de novo* synthesis of fatty acids. ArKO male mice on the control diet had significantly elevated levels of

expression compared to WT mice on the control diet, (p=0.026, Fig 6A). There were no changes in expression in females (Fig 6B).

Fatty acid synthase (FAS), another enzyme involved in *de novo* synthesis of fatty acids was also up regulated in ArKO males on the control diet compared with WT controls, (p=0.038, Fig 6C). Females showed no changes in its expression (Fig 6D).

Acetyl CoA carboxylase β (ACCβ) is an enzyme that may have a specific role in regulation of β-oxidation due to its ability to inhibit carnitine:palmitoyl-CoA acytransferase 1 (CPT1) (24). In both males and females there were no change in expression of accβ, regardless of genotype and diet (Fig 6E and F).

Expression of genes involved in the export of triglycerides out of the liver

Apolipoprotein E (apoE) is an apolipoprotein, which is a key component of very low-density lipoprotein (VLDL), which is the major vehicle for the export of triglycerides out of liver. Its expression was unchanged in male or female mice livers regardless of genotype or diet (Fig 7A and B). Microsomal triglyceride transfer protein (MTTP) catalyses the transfer of lipids to the apolipoprotein B, which is and the key component of VLDL (25). Preliminary data to determine the levels of MTTP transcript revealed no changes in expression between male ArKO and WT mice (data not shown).

Expression of genes involved in fatty acid uptake

ArKO males on the control diet had significantly up-regulated expression of adipocyte differentiated related protein (ADRP) compared to WT control mice, (p=0.007, Fig 8A). No significant changes were seen between genotypes on the 2% cholesterol diet (p=0.069, Fig 8A). Cholesterol fed ArKO and WTs had a significant increase in expression of ADRP compared to genotype matched controls (p=0.049, p=0.021,

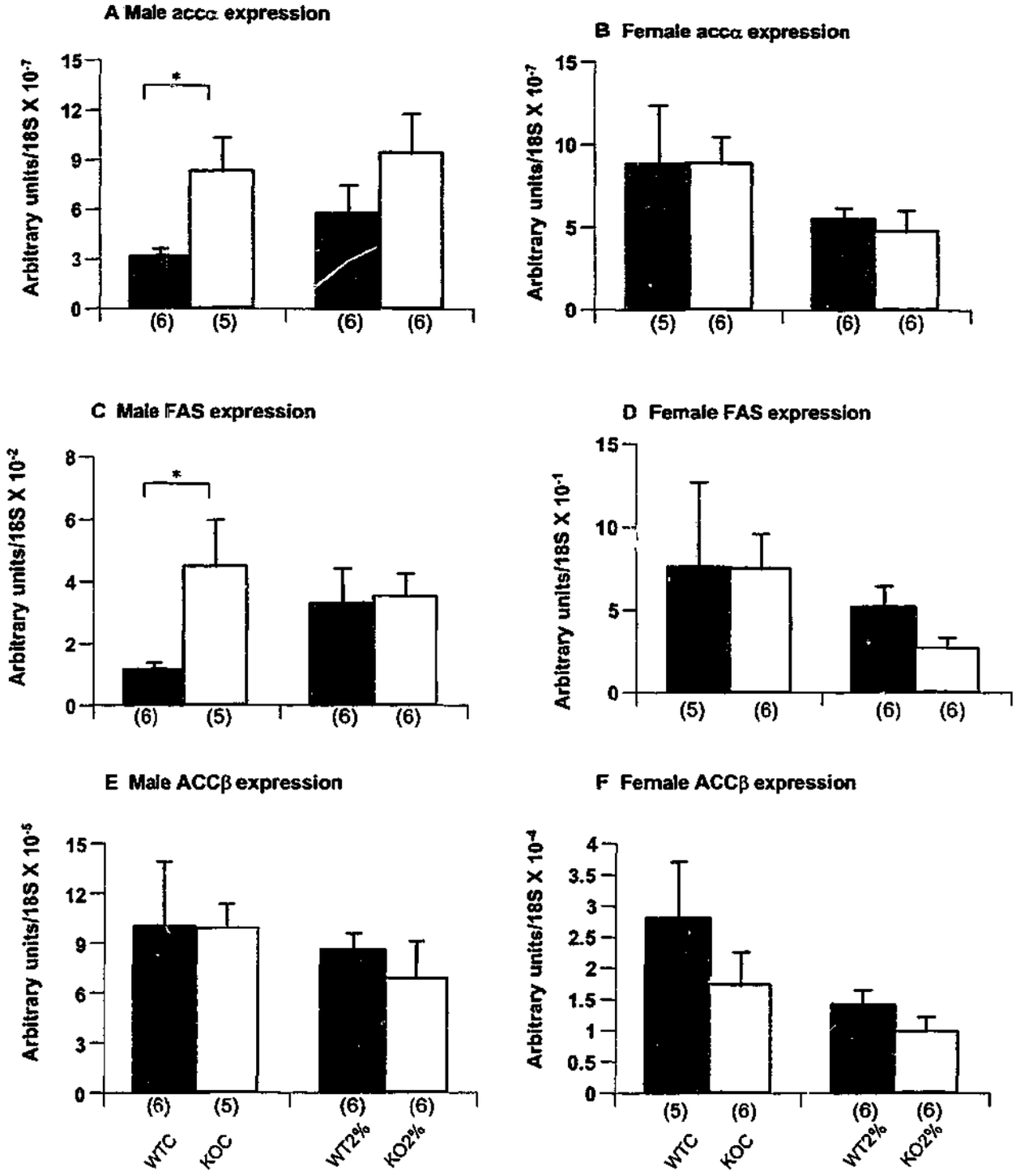


Figure 6. Levels of transcripts for genes regulating fatty acid synthesis.
A Male ACCα transcript levels. ArKO male mice on the control had significantly elevated levels of expression of ACCα compared with WT control mice, *p<0.05. **B Female ACCα transcript levels.** NS changes in expression. **C Male FAS transcript levels.** Control ArKO male mice had significantly elevated transcript levels of FAS compared with WT control mice, *p<0.05. **D Female FAS transcript levels.** NS changes in expression. **E Male ACCβ transcript levels.** NS changes in expression. **F Female ACCβ transcript levels.** NS changes in expression.
ArKO (KO) white bar, WT black bar. C refers to control diet and 2% refers to 2% cholesterol diet.

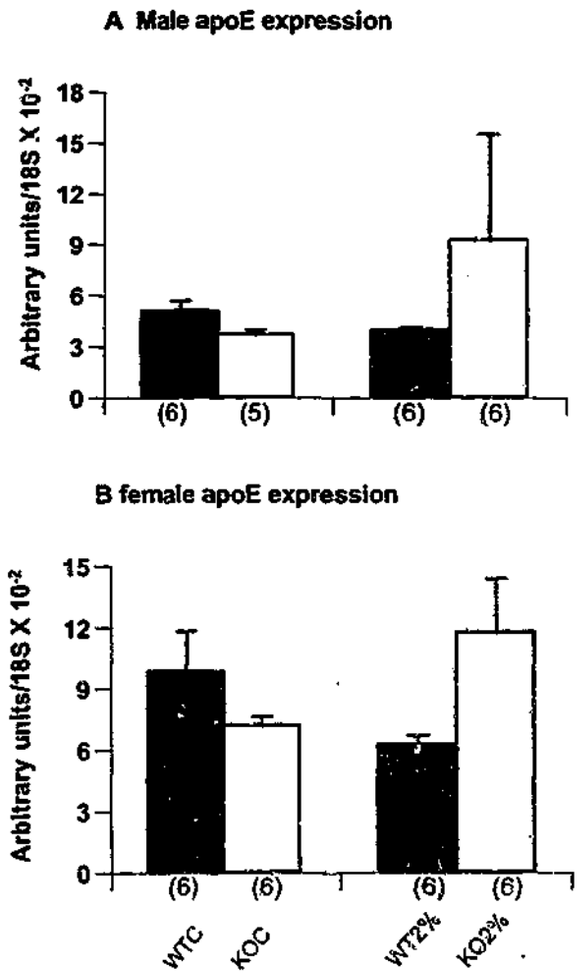
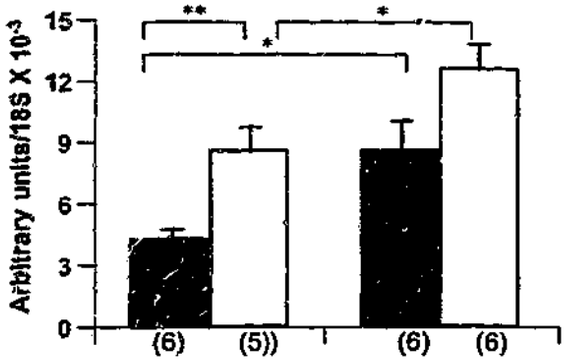
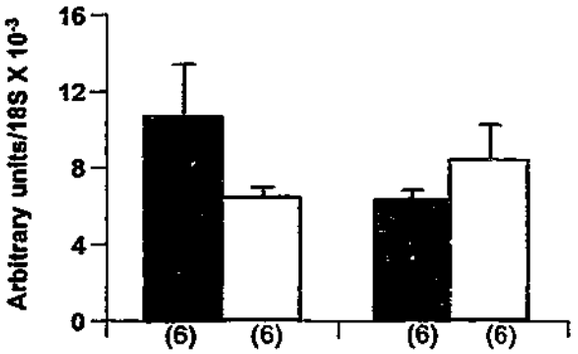


Figure 7. Levels of transcripts for apo E
A Male apo E transcript levels. NS change in expression. **B Female apo E transcript levels.** NS change in expression.
ArKO (KO) white bar, WT black bar. C refers to control diet and 2% refers to 2% cholesterol diet.

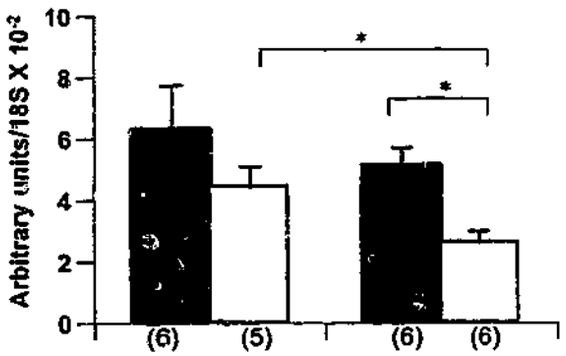
A Male ADRP expression



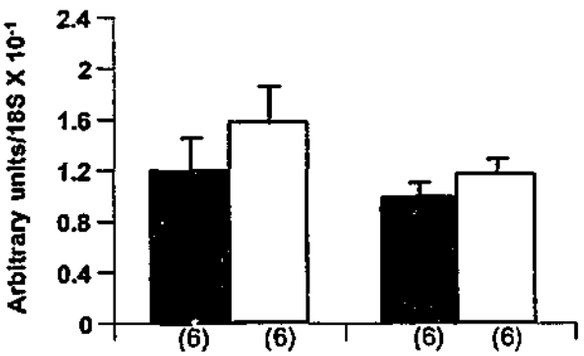
B Female ADRP expression



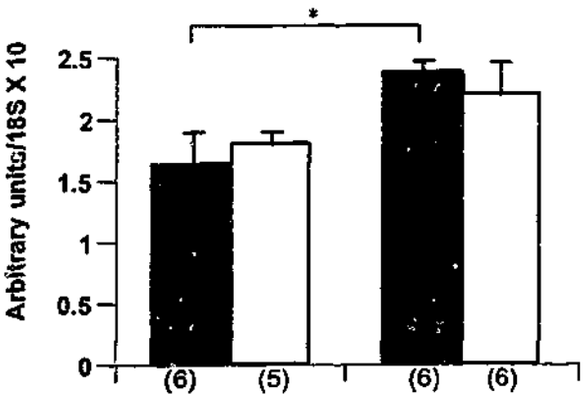
C Male CD36 expression



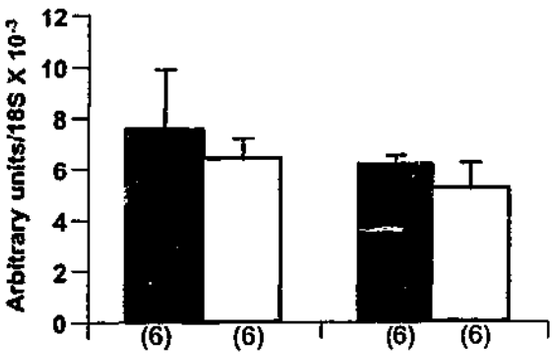
D Female CD36 Expression



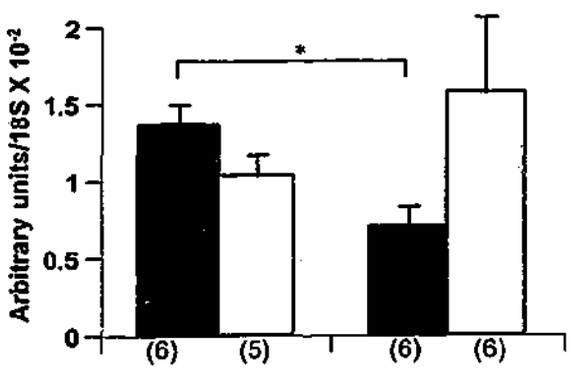
E Male FATP2



F Female FATP2



G Male FATP5



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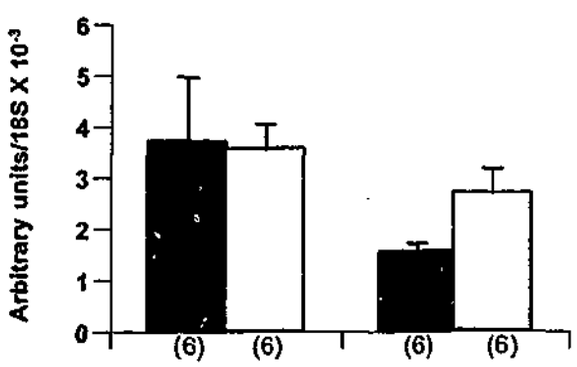

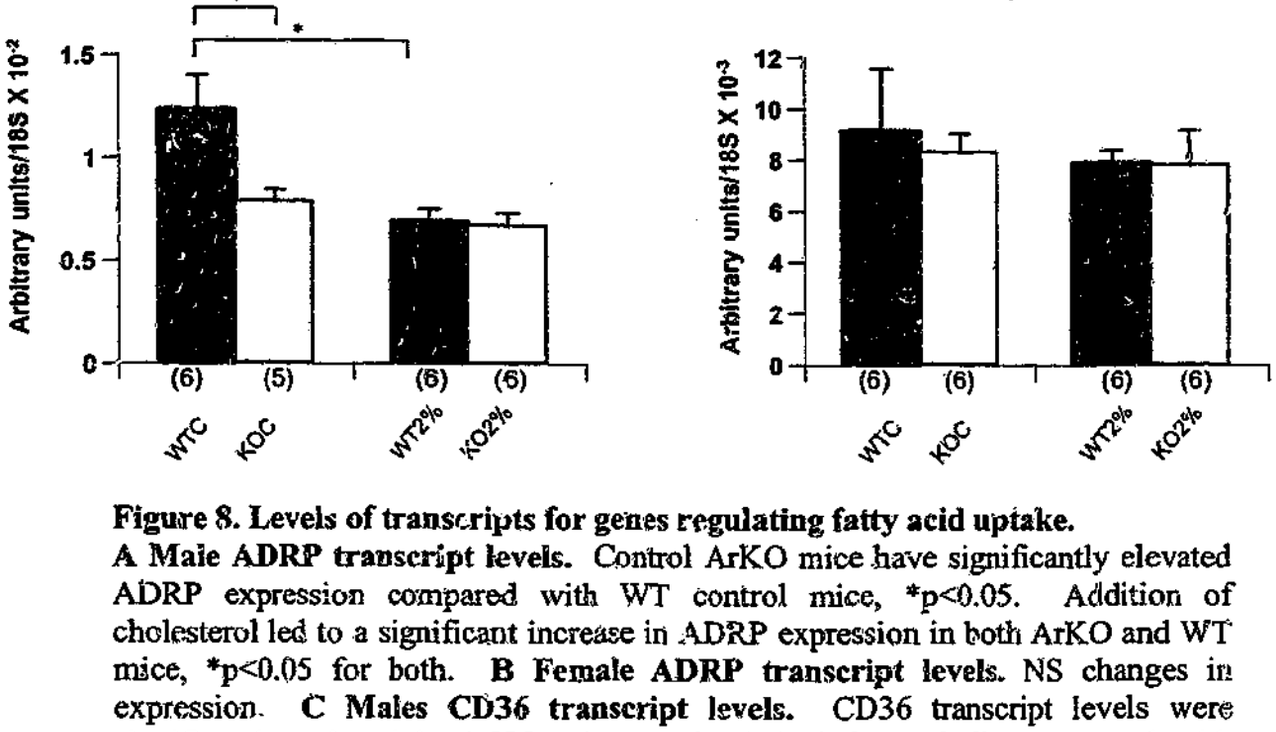

Figure 8. Levels of transcripts for genes regulating fatty acid uptake.
A Male ADRP transcript levels. Control ArKO mice have significantly elevated ADRP expression compared with WT control mice, *p<0.05. Addition of cholesterol led to a significant increase in ADRP expression in both ArKO and WT mice, *p<0.05 for both. **B Female ADRP transcript levels.** NS changes in expression. **C Males CD36 transcript levels.** CD36 transcript levels were significantly reduced in ArKO mice on the 2% cholesterol diet compared with ArKO control mice, *p<0.05 and significantly reduced compared with diet matched WTs, p<0.01. **D Female CD36 transcript levels.** NS changes in expression. **E Males FATP2 transcript levels.** WT mice expression increased when fed the 2% cholesterol diet compared WT control mice, P<0.05. **F Female FATP2 transcript levels.** NS changes in expression. **G Male FATP5 transcript levels.** WT mic expression levels significantly decreased upon cholesterol feeding, P<0.05. **H Female FATP5 transcript levels.** Trend for a reduction in expression between control mice and those on the 2% cholesterol diet, p=0.051. **I Male SR-B1 transcript levels.** Control ArKO mice had significantly reduced levels expression compared with WT control mice, *p<0.05. WT mice fed the 2% cholesterol diet had significantly reduced levels of expression compared with WT control mice, *p<0.05. **J Female SR-B1 transcript levels.** NS changes in expression.
ArKO (KO) white bar, WT black bar. C refers to control diet and 2% refers to 2% cholesterol diet.

respectively; Fig 8A). Conversely, females showed no changes in ADRP expression regardless of genotype or diet (Fig 8B).

CD36, another fatty acid transporter, showed no difference in levels of expression between ArKO and WT male mice on the control diet. ArKO male mice had significantly reduced expression when fed the 2% cholesterol diet compared to ArKO male mice fed the control diet ($p=0.037$, Fig 8C) and compared to diet matched WT male mice ($p=0.005$, Fig 8C). Females showed no changes in expression regardless of genotype or diet (Fig 8D).

Fatty acid transporters 2 and 5 (FATP2, FATP5) were also measured. Males had no significant differences in FATP2 expression between genotypes on either the control or 2% cholesterol diet. Expression of FATP in WT mice increased when fed the 2% cholesterol diet compared with WT on the control diet ($p=0.026$, Fig 8E). ArKO males showed no change in expression of FATP2 between diets. Again females showed no differences in FATP2 expression, regardless of genotype or diet (Fig 8F).

Similar to FATP2 expression, FATP5 expression in WT male mice was reduced when fed a diet with 2% cholesterol ($p=0.005$, Fig 8G). Female mice had reduced levels of FATP5 expression between control mice and those on the 2% cholesterol diet, however this was not significant ($p=0.051$, Fig 8H).

A scavenger B receptor class 1 (SR-B1), which is involved in transport of cholesterol and fatty acids, was also measured. The male ArKO mice on the control diet had significantly lower levels of expression compared with WT control mice ($p=0.048$, Fig 8I). There were no differences between ArKO and WT mice when fed the 2% cholesterol diet. There was also a significant decrease in expression for WT mice fed the 2% cholesterol diet compared with the WT controls ($p=0.013$, Fig 8I). Female mice showed no changes in expression (Fig 8J).

Discussion

Estrogen deficiency leads to obesity in both male and female ArKO mice (8), but only the males develop hepatic steatosis. From the data presented here, it appears that the hepatic steatosis in the ArKO male mice is a result of elevated levels of hepatic triglycerides, although we have previously shown that there is also a modest increase in the cholesterol content of the male ArKO livers (12). Gene expression studies revealed increased lipogenesis, indicated by increases in FAS and accα expression and an increase in fatty acid transport as seen by elevated ADRP expression. There was no change in the expression of apoE, hence it is likely that there was no compensatory increase in VLDL production for export of triglycerides from the liver thereby exacerbating the phenotype. Most interestingly, cholesterol feeding reversed the obese phenotype of the ArKO mice and also the hepatic steatosis in the ArKO males. Results of the study are summarised in Table 2.

Effects of Estrogen

Previously, we reported that estrogen deficiency in ArKO mice leads abdominal obesity (8) and increased lipoprotein lipase (LPL) expression in adipose tissue (11). LPL catalyses hydrolysis of serum triglycerides releasing free fatty acids (FFA) and sn2-monoglycerides for uptake into the adipose tissue (26), whereas hormone sensitive lipase (HSL) catalyses hydrolysis of intracellular triglycerides for release of FFA. (25). As these free fatty acids are from a central adipose depot rather than a peripheral adipose depot they are able to drain directly into the portal vein (25). This rise in hepatic FFA can provide substrate for increased triglyceride synthesis in the liver (27,28). Additionally, in ArKO males there is also elevated FAS and accα expression, resulting in increased hepatic fatty acid production, thus contributing further to the

Table 2. Summary of Results

	Absence of Estrogen		**2% cholesterol diet**			
Parameters Measured	M	F	M		F	
			KO	WT	KO	WT
Body Weight	⇑	⇑	⇓	⇔	⇓	⇔
Liver Weight	⇑	⇔	⇓	⇔	⇔	⇔
Hepatic Steatosis	⇑	⇔	⇓	⇔	⇔	⇔
Hepatic Triglycerides	⇑	⇔	⇓	⇔	⇑	⇑
Serum Triglycerides	⇔	⇑	⇓	⇔	⇓	⇔
accα	⇑	⇔	⇔	⇔	⇔	⇔
FAS	⇑	⇔	⇔	⇔	⇔	⇔
accβ	⇔	⇔	⇔	⇔	⇔	⇔
apoE	⇔	⇔	⇔	⇔	⇔	⇔
ADRP	⇑	⇔	⇑	⇑	⇔	⇔
CD36	⇔	⇔	⇓	⇔	⇔	⇔
FATP2	⇔	⇔	⇔	⇑	⇔	⇔
FATP5	⇔	⇔	⇔	⇓	⇔	⇔
SR-B1	⇓	⇔	⇔	⇓	⇔	⇔

Absence of estrogen refers to ArKO compared to WT controls, 2% cholesterol diet compared with control diet, M=male, F=female, KO=ArKO, WT=wildtype

fatty liver. Insulin inhibits HSL activity in the adipose tissue. However, in this situation of abdominal obesity, the ability of insulin to inhibit lipolysis is impaired (29-32) and may lead to further secretion of FFA from adipose tissue (25), thereby leading to further production of lipids in the liver.

Transport of fatty acids into cells occurs by two main mechanisms, passive diffusion and facilitated transfer (33). It is probable that facilitated transfer is the main mechanism of FFA uptake since passive diffusion would be unlikely to account for observed uptake rates (34,35). ADRP is known to enhance the uptake of long chain fatty acids (LCFA), but not medium or short chain FA (36). Increased ADRP expression suggests that LCFA uptake is increased in the estrogen-deficient males. On the contrary, estrogen deficiency in females had no effects on expression of mRNA encoding proteins associated with fatty acid uptake despite elevated levels of FFA and serum triglyceride levels, though it is possible that these changes are post-transcriptional.

CD36 is a fatty acid transporter, which belongs to the SR-B1 family of scavenger receptors and facilitates uptake of FFA and oxidized low density lipoproteins (37). This transporter has been shown to have sexually dimorphic properties, with higher protein expression in females (38), indicating a role for sex steroids in its regulation. In this study, neither estrogen status nor gender had effects on mRNA levels for CD36, possibly suggesting that estrogen regulates this transporter at the post-transcriptional level. Conversely, SR-B1, another scavenger receptor primarily involved in cholesterol transport, is down regulated in the absence of estrogen, indicated by lower levels of expression observed in ArKO male mice on the control diet compared with WT control mice.

Also important for triglyceride regulation in liver is the export of triglycerides from the liver through VLDL secretion. Hepatic VLDL production is predominantly substrate

driven and the rate limiting step is FFA availability for new triglyceride synthesis (39). Apolipoprotein B (apoB) is an important component of the VLDL, however its regulation occurs post-transcriptionally (40) hence, its transcripts are not an ideal marker for VLDL synthesis. ApoE is another apolipoprotein present on VLDL, apoE deficient mice have been shown to have an impaired ability to secret VLDL (40). ApoE is regulated at the level of transcription and thus its transcript levels are a suitable marker. Estrogen deficiency did not lead to a change in apoE expression, likely reflecting no change in VLDL production. For these reasons, the data suggests that the livers of the male ArKO mice are unable to counteract the elevated fatty acid production and uptake with increased VLDL output, thereby leading to a fatty liver phenotype (28). Additionally, Toda *et al* (41) showed in their male ArKO mouse model that VLDL fractions from ArKO plasma were no different from those from WT mice.

We have previously shown that cholesterol homeostasis is altered in the livers of ArKO mice in a sexually dimorphic manner (12). We show here that this sexual dimorphism is extended to triglyceride homeostasis. The rodent liver is known to have sex specific properties due to differing patterns of growth hormone secretion which may lead to differential expression of cytochrome P450s (42,43). Previous work in male mice has demonstrated that, pulsatile secretion of GH leads to activation of JAK kinases, which in turn phosphorylate Stat5b transcription factor, allowing it to translocate to the nucleus to activate target genes (44-46). Interestingly, Stat5b null mice have pale and enlarged livers (44), similar to the ArKO male mice. Thus it is possible that growth hormone deficiency may lead to hepatic steatosis via a mechanism involving Stat5b. In this context, growth hormone deficient men have presented with hepatic steatosis (47) and this was reversed by growth hormone replacement in one

man (48). Whether or not growth hormone related mechanisms are involved in the hepatic steatosis of the male ArKO mice is currently under investigation.

Other models of hepatic steatosis are the leptin resistant and deficient mouse models. Leptin is a peptide hormone that is secreted from adipose tissue, which acts in a negative feedback fashion on the leptin receptors that are present in the hypothalamus. Leptin is known to regulate food intake and spontaneous physical activity (49) and more recently has been shown to inhibit lipogenesis, cholesterol synthesis and to stimulate fatty acid oxidation (50). Estrogen deficiency in males but not females leads to cell death occurring in the arcuate nucleus (Arc) and medial preoptic area (MOP) regions of the hypothalamus (51). The Arc and medial eminence (ME) regions contain the highest concentration of leptin receptors in the brain (52) hence it is possible that leptin signalling is reduced in the male ArKO mice, contributing to the sexually dimorphic lipid phenotype in the ArKO mice.

Effects of a High Cholesterol Diet

Previously, the ArKO mice were challenged with a high cholesterol diet in order to examine the effects of estrogen on cholesterol homeostasis (12). Surprisingly, the high cholesterol diet led to a reversal of the obese phenotypes in the ArKO mice and the hepatic steatosis in the ArKO male mice. Estrogen deficiency led to an elevation in genes regulating lipogenesis in male mice, whereas cholesterol feeding had no significant effect on genes involved in lipogenesis in either sex. Cholesterol feeding had different effects on the fatty acid transporters in male mice depending on estrogen status. Cholesterol feeding had an effect on ADRP expression only in estrogen deficient males, where it was increased. Expression of another transporte CD36 was reduced by cholesterol feeding only in estrogen-deficient males. Conversely, the transporters FATP2 and FATP5 were only altered by cholesterol feeding in estrogen-replete males. Sexually dimorphism was also observed here, in that FATP5 expression

was reduced in females after cholesterol feeding regardless of estrogen status. Hence estrogen and gender play important roles in affecting the ability of cholesterol to regulate fatty acid transport. These changes however, do not explain the mechanisms which have led to the reversal of the hepatic steatosis in the ArKO males. As proposed earlier, the hepatic steatosis is secondary to the obesity and insulin resistance and therefore it is possible that cholesterol is having its effects on the adipose tissue to reverse the obese phenotype (53), and hence prevent the hepatic steatosis.

In conclusion, we have demonstrated a role for estrogen in the regulation of triglyceride metabolism in the livers of male but not female mice, indicating a sexually dimorphic regulation of this important homeostatic pathway.

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Chapter Three: Estrogen Replacement Reverses the Hepatic Steatosis Phenotype in the Male Aromatase Knockout (ArKO) Mouse.

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Declaration for Thesis Chapter Three

In the case of chapter three, contributions to the work involved the following:

Name	% contribution	Nature of Contribution
Kylie Hewitt	90%	Collection of liver and processing all tissues and performing all assay Preparation of manuscript
Kyriakos Pratis	3%	Performed Injections all mice
Margaret Jones	2%	Supervisor and collection of fat pad masses and weighing them
Evan Simpson	5%	Supervisor and editorial assistance

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Signature 3		31/10/03
Signature 4		30/10/03

Estrogen replacement reverses the hepatic steatosis phenotype in the male aromatase knockout (ArKO) mouse

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Key words: estrogen, aromatase, hepatic steatosis, triglycerides

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Abstract

The aromatase knockout (ArKO) mouse cannot synthesize endogenous estrogens due to a disruption to the *Cyp19* gene. Previously, we have reported that both male and female ArKO mice present with an age progressive obese phenotype, and there is a sexually dimorphic disruption to hepatic cholesterol and triglyceride homeostasis. Only ArKO males have elevated hepatic triglyceride levels leading to hepatic steatosis due in part to an increase in expression of enzymes involved in *de novo* lipogenesis and transporters involved in fatty acid uptake. In this study ArKO males were treated with 17β-estradiol (E_2) (3 μg/ kg/ day) at 18 weeks old for 6 weeks. WT controls were not treated and ArKO controls received vehicle oil injections. Estrogen replacement led to a reversal of the previously reported obese and fatty liver phenotypes; this was achieved by reductions in gonadal, visceral and BAT weights and a significant decrease in hepatic triglyceride levels. Estrogen deficiency led to a significant up-regulation of hepatic fatty acid synthase expression which was reduced with E_2 replacement, although not quite reaching significance. Acetyl CoA carboxylase α showed no significant changes. Adipocyte differentiated regulatory protein, a fatty acid transporter, was significantly elevated in estrogen deficient males and E_2 replacement significantly reduced these levels. Scavenger receptor class b type I showed no significantly changes. This study reveals that the previously reported disruption to triglyceride homeostasis in estrogen deficient males can be reversed with E_2 treatment, indicating an important role for estrogen in maintaining triglyceride and fatty acid homeostasis in males.

Introduction

Hepatic steatosis is linked to insulin resistance (IR) and the 'metabolic syndrome' (1,2). Features of the metabolic syndrome include central obesity rather than peripheral obesity, dyslipidemia, characterised by elevated triglycerides, low-density lipoproteins (LDL) and reduced high-density lipoproteins (HDL) (3). Menopausal women have a reduction in circulating estrogen levels and this coincides with a shift in body fat from the gluteal to the abdominal region, thus linking lack of estrogen to central obesity (3). In addition to this shift in body fat, postmenopausal women have elevated circulating triglyceride levels compared with premenopausal women, and men in general are also known to have higher circulating levels of triglycerides compared with women, again establishing a strong relationship between estrogen and triglyceride levels.

Mouse models of estrogen deficiency also present with dyslipidemia. Specifically, the aromatase knockout (ArKO) mouse (4) presents with central obesity, hypercholesterolemia, hyperinsulinemia, hyperleptinemia and hypertriglyceridemia (5) and importantly the male mice have hepatic steatosis (5,6). Estrogen receptor α knockout (αERKO) mice and the estrogen receptor α and β double knockout (αβERKO) mice (7,8) also display obese phenotype similar to the ArKO mice (5), but the hepatic phenotype of these animals has not been reported. The estrogen receptor β knockout (βERKO) mouse does not have a lipid phenotype (8). Thereby suggesting ERα is the more important ER in lipid homeostasis.

We have reported that the hepatic steatosis present in ArKO males is due to an accumulation of hepatic triglycerides. Molecular characterisation of this phenotype revealed that estrogen deficiency in males led to elevated fatty acid synthase (FAS) and acetyl CoA carboxylase α (ACCα) expression. There was also an increase in

expression of adipocyte differentiated related protein (ADRP), a fatty acid transporter present in the liver (6). There was no change in apoE expression, suggesting there was no compensatory increase in very low-density lipoprotein (VLDL) secretion, which would allow an increase in hepatic triglyceride clearance, thus further exacerbating the phenotype (6). Additionally, we failed to observe an increase in fatty acid β-oxidation. As well as elevated hepatic triglycerides in estrogen deficient male mice, previously we have reported that male ArKO mice also have elevated levels of hepatic cholesterol (9). In addition to our model, Toda *et al* (10) generated another ArKO mouse by disrupting exon 9 of the *Cyp19* gene. They reported the presence of hepatic steatosis due to a disruption in β-oxidation, shown at the level of gene expression as well as the catalytic activity of these enzymes. This phenotype was reversed by 17β-estradiol treatment (11). However they did not indicate if this was sexually dimorphic.

Human models of aromatase deficiency have also been identified (12-16), and like the ArKO mouse they also present with dyslipidemia. In one male patient hepatic steatosis was described which was reversed with 17β-estradiol treatment (15).

Therefore the aim of this study was to attempt to rescue the fatty liver phenotype in the male ArKO mouse with 17β-estradiol treatment.

Methods

Mice

The ArKO mice were generated by deleting 90% of exon 9 of the *Cyp*19 gene as described by Fisher *et al* (4). WT and homozygous null offspring were generated by heterozygous matings. The genotype of the offspring was determined by PCR as described by Robertson *et al* (17). The animals were housed in specific pathogen-free

conditions and had unlimited access to drinking water and food. These studies were approved by the Monash Medical Centre Animal Ethics Committee.

Diets

Soy free mouse chow (Glen Forest stock feeders, Perth, Australia) is the diet used to feed the mice; it contains no soy products, which are found in regular mouse chow, as soy is known to have estrogenic effects (18). This diet contains 15% of calories as fat (0.02% cholesterol), 20% calories as protein and 65% of calories as carbohydrate.

Hormone Preparation

17β-estradiol (Sigma, St Louis, MO) was dissolved in methylene chloride (Unilab, New Zealand) and added to peanut oil (methylene chloride:oil, 1:2 v/v). The methylene chloride was evaporated by bubbling with nitrogen and warming the solution to 37 °C. Vehicle oil injections were identically prepared, omitting 17β-estradiol.

Experimental Design

ArKO males received daily subcutaneous injections of 17β-estradiol (3 μg/ kg/ day) from 18 weeks of age for 6 weeks. WT controls did not receive any treatment and ArKO controls received vehicle oil injections.

Tissue Collection and Histology

Animals were weighed, then killed at 24 weeks by CO_2 asphyxiation. Blood was removed by cardiac puncture and stored at -20°C. The liver was removed, weighed and part of it snap frozen in liquid nitrogen and stored at -80°C for gene and lipid analysis. Part of the liver was immersion fixed in Bouins fixative then stored in 70 % ethanol. Fixed samples were embedded in a random orientation in paraffin and sliced into 7 μm sections. Sections were then stained with hematoxylin, counterstained with eosin and

coverslipped with DPX (BDH, UK). Gonadal, visceral and brown adipose tissue (BAT) pad were also removed and weighed.

Measurement of Hepatic Triglycerides and Cholesterol

Hepatic triglycerides and cholesterol were extracted from liver by homogenising 0.2g of tissue in 10ml of chloroform/methanol (2:1v/v) (19). Samples were centrifuged for 20min at 800g, the lipid phase removed and the chloroform evaporated. Triglyceride and cholesterol were quantified using Infinity™ Triglycerides Lipid Stable Reagent (Thermo Trace, Melbourne, Australia) and Cholesterol Lipid Incorporating Dynamic Stabilization Technology, (Thermo Trace, Melbourne, Australia), respectively. Triglyceride and Cholesterol Calibrators were used as references (Sigma USA).

Gene Analysis

RNA was extracted from liver using the phenol-chloroform method (Ultraspec RNA, Fisher Biotech, Australia) and quantified spectrophotometrically. RNA quality was assessed on an ethidium bromide (Sigma, USA) agarose (Promega, Life Sciences, USA) gel. Two-step RT-PCR was performed using random primers (Roche, Germany) and AMV reverse transcriptase enzyme (Promega Life Sciences, USA). A Lightcycler™ (Roche, Germany) was used to quantitate mouse transcripts using specific primer pairs (6). Primer pairs were shown to be specific through single peak melting curves and a single product was seen on an ethidium bromide (Sigma, USA) agarose (Promega, Life Sciences, USA) gel corresponding to the appropriate product size as measured by a 1kb ladder (Promega, Life Sciences, USA).

All samples were normalized to 18S. All samples were run individually in three separate reverse transcription reactions and transcripts measured using real time PCR.

The data was presented as the mean of three consistent runs. Inter-assay variability was assessed using reference cDNA from a control animal in repeated runs.

Statistical Analysis

All graphs were expressed as means ±SEMs and statistics were preformed using ANOVA, SPSS for Windows version 10.0 (USA). A minimum of five animals were used per group.

Results

Body Weight

Although ArKO male mice were slightly heavier weight than the WT control mice this did not reach statistical significance. However, following estrogen replacement (E_2), the body weight of the ArKO mice returned to the weight of the WT control mice ($p=0.002$, Fig 1A).

Liver Weight

Control ArKO mice had a significantly heavier liver weight compared with WT control mice ($p=0.026$, Fig 1B). E_2 led to a reduction in ArKO liver weight to levels comparable with WT controls ($p=0.003$, Fig 1B).

Fat pad weight data

ArKO male control mice had significantly heavier gonadal fat pad weight compared with WT control mice ($p=0.038$; Fig 1C). E_2 replacement did not significantly reduce gonadal fat pad weight in the ArKO mice ($p=0.111$, Fig 1C).

ArKO control mice also had significantly heavier visceral fat pad weight compared with WT control mice ($p=0.027$, Fig 1D) and E_2 treatment significantly reduced visceral fat pad weight in the treated ArKO mice ($p=0.04$, Fig 1D).

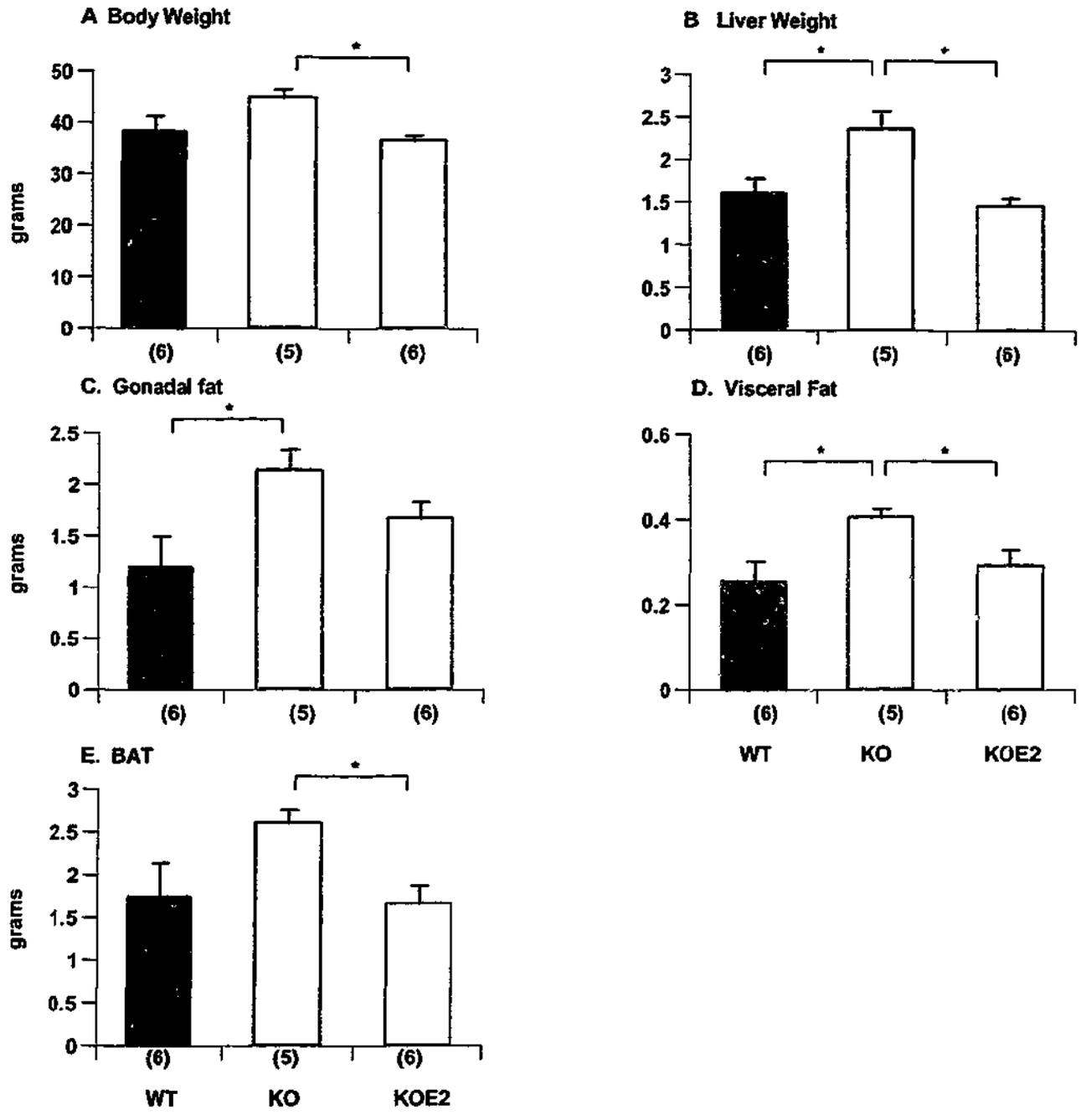

Figure 1: Organ Weight Data

A Body Weight, there was NS difference WT and ArKO mice, and E_2 replacement significantly reduced body weight in ArKO mice, *$p<0.05$. **B Liver Weight**, ArKO mice have significantly elevated liver weight compared with WT mice, *$p<0.05$, which is significantly reduced with E_2 replacement, *$p<0.05$. **C Gonadal Fat Pad Weight**, ArKO mice have significantly heavier fat pad weight compared with WT mice, *$p<0.05$. NS change with E_2 replacement. **D Visceral Fat Pad Weight**, ArKO mice significantly heavier than WT, E_2 replacement significantly reduced this, *$p<0.05$. **E BAT**, NS differences between controls, and E_2 replacement significantly reduced BAT weight, *$p<0.05$.

ArKO (KO) white bars, WT black bars, E_2 estrogen replacement.

There were no significant differences between ArKO and WT male mice in BAT weight (p=0.098, Fig 1E) however E_2 replacement in the ArKO mice lead to a significant reduction in BAT weight compared with ArKO control mice, (p=0.008, Fig 1E).

Liver Morphology

ArKO male mice have an accumulation of lipids in their livers (Fig 2B) which are not apparent in the WT male mice (Fig 2A). Following E_2 replacement there was a reversal of the fatty liver phenotype (Fig 2C).

Hepatic Triglyceride and Cholesterol Levels

ArKO control mice had significantly increased levels of hepatic triglyceride levels compared with WT controls (p=0.000, Fig 3A). These levels were significantly reduced with E_2 treatment (p=0.000, Fig 3A).

ArKO control mice had significantly elevated hepatic cholesterol levels compared with WT control mice, (p=0.003, Fig 3B), however, E_2 replacement in the ArKO mice did not significantly affect these levels (p=0.558, Fig 3B).

Expression of transcripts encoding enzymes involved in *de novo* synthesis of fatty acids

To explain the changes in body, fat and liver weight; hepatic triglyceride levels following E_2 replacement, liver samples were analysed for expression of various genes, which have previously been shown to the altered in ArKO male mice.

ArKO control mice showed a significant up-regulation of FAS expression compared with WT mice (p=0.004). With E_2 treatment there was a decrease in FAS expression in the ArKO mice (p=0.096, Fig 4A), although this did not reach significance.

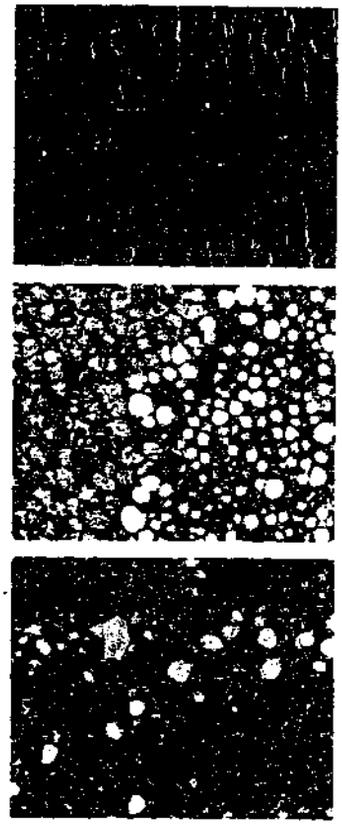

Figure 2: Liver Morphology
A WT mice have no lipid droplet. B ArKO mice have lipid droplets. C ArKO mice E_2 replacement reduced lipid droplets compared control ArKOs. Scale Bar 100μm.

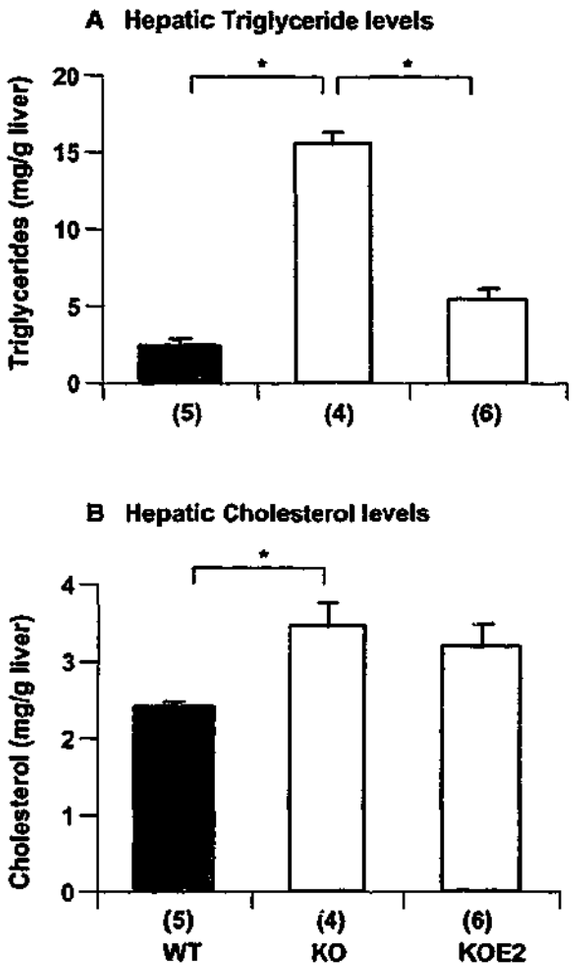

Figure 3: Hepatic Lipid Levels.
A Hepatic Triglyceride levels ArKO levels were significantly higher compared with WT, $p<0.05$ and E_2 replacement significantly reduced these levels, *$p<0.05$. **B Hepatic Cholesterol levels**, ArKO levels were significantly higher compared with WT, $p<0.05$, NS change with E2 replacement.
ArKO (KO) white bars, WT black bars, E_2 estrogen replacement.

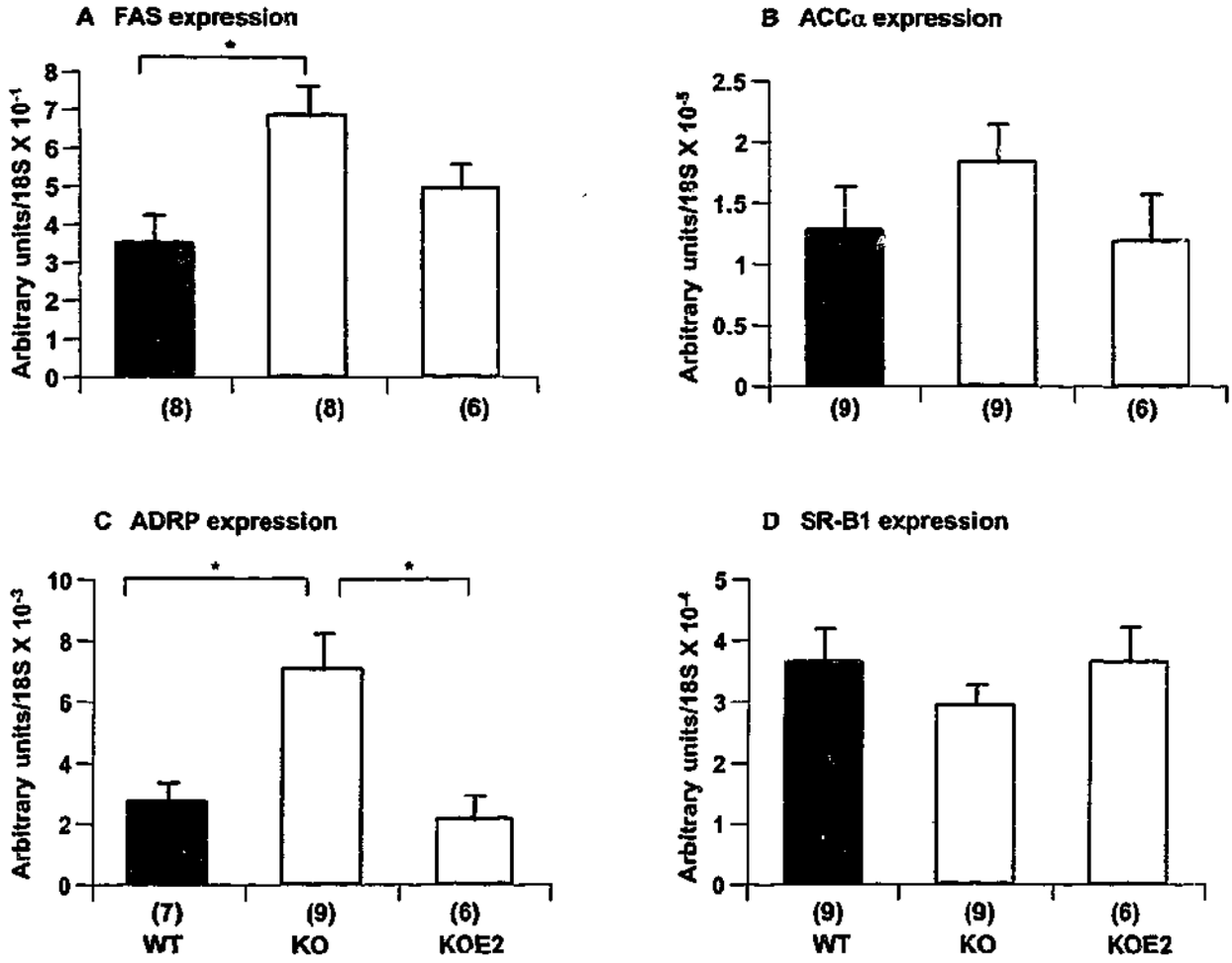

Figure 4: Gene expression data

A FAS expression ArKO levels were significantly higher compared with WT, *$p<0.05$. **B ACCα**, NS changes. **C ADRP expression** ArKOs levels were significantly elevated compared with WT, *$p<0.05$, E_2 treatment significantly reduced expression in ArKOs, *$p<0.05$. **D SR-B1 expression** NS changes.

ArKO (KO) white bars, WT black bars, E_2 estrogen replacement.

ACCα, another key enzyme involved in *de novo* synthesis of fatty acids, showed similar changes however did not reach the level of significance between any of the groups measured, (Fig 4B).

Gene Expression of fatty acids transporters

ADRP, a fatty acid transporter, was significantly up-regulated in the ArKO male mice compared to WT control mice ($p=0.005$), additionally, there was also a significant decrease in its expression following the E_2 replacement in ArKO mice ($p=0.003$, Fig 4C).

SR-B1 is also involved in uptake of fatty acids and cholesterol uptake but showed no significant differences between any of the groups (Fig 4D).

Discussion

We have previously shown that estrogen deficiency in male mice leads to hepatic steatosis (5,6). In the present study we demonstrate that this phenotype can be rescued by E_2 replacement. This treatment significantly reduced hepatic triglycerides, with a concomitant decrease in lipid droplets; conversely E_2 replacement had little effect on hepatic cholesterol levels. Additionally, estrogen deficiency led to an increase in transcripts encoding factors involved in lipogenesis and long chain fatty acid (LCFA) uptake in the male mice and the levels of these transcripts were normalised by E_2 replacement. Not only did the E_2 replacement have positive effects on the liver but it also reduced the body weight, visceral pad weight, BAT weight and a trend for a reduction in gonadal fat pad weight, indicating a general effect of E_2 to reduce lipid accumulation. The results are summarised in Table 2.

Table 1. Summary of Results

Parameters measured	*Absence of estrogen*	*E_2 replacement*
Body Weight	⇔	⇓
Liver Weight	⇑	⇓
Gonadal fat pad Weight	⇑	⇔
Visceral Fat pad Weight	⇑	⇓
BAT	⇔	⇓
Fatty liver	⇑	⇓
Hepatic Triglycerides	⇑	⇓
Hepatic Cholesterol	⇑	⇔
ACCα	⇔	⇔
FAS	⇑	⇔
ADRP	⇑	⇓
SR-B1	⇔	⇔

Absence of estrogen refers to ArKO mice compared to WT mice, E_2 replacement refers to ArKO replaced with E_2 compared with ArKO controls.

There is an increasing body of evidence to suggest that there is a link between hepatic steatosis, insulin resistance (IR) and obesity (2,20). A key defect in IR is central adipose tissue resistance to insulin-mediated suppression of lipolysis (21). A consequence of this resistance is elevated free fatty acid (FFA) levels (21). FFA from central adipose depots are able to drain directly into the portal blood and promote hepatic gluconeogenesis which increases hepatic glucose output (2,21), stimulating the pancreatic β cells to increase insulin secretion, which up-regulates lipogenesis in the liver (21). Insulin also acts to inhibit lipolysis; however when there is central obesity these actions are impaired. Thus a positive loop of disregulated lipid homeostasis is generated, eventuating in hepatic steatosis.

Estrogen deficiency in the male mice leads to hepatic steatosis, probably due in part to an increase in lipogenesis indicated by increases in FAS and accα expression; E_2 replacement lowered the expression of FAS. Despite these findings, other studies have shown estrogen to be a stimulator of lipogenesis in chick livers (22,23); in the rat liver (24), and in male *Xenopus laevis* (25); although in ewes E_2 treatment inhibited lipogenesis (26), this was however in adipose tissue rather than in the liver.

LCFA uptake may also be elevated in the state of estrogen deficiency as seen by increased levels of ADRP expression. This is reversed with E_2 replacement. The mechanism whereby estrogen regulates ADRP expression is not yet understood, nevertheless another transporter, FAT/CD36 is also affected by sex steroids (27). These studies reveal a possible involvement of estrogen in the regulation of FFA transport.

Adipose tissue is believed to have a buffering action by suppressing the release of FFA into the circulation and by increasing triglyceride clearance (28). In the circumstance of obesity, adipose tissue function is altered so that buffering is less effective and the

adipocytes are filled, hence resisting further fat storage (28). Conversely, rodents who have lipodystrophy also present with IR and diabetes (29-31). This lack of adipose tissue prevents any buffering of FFA, also contributing to the accumulation of triglycerides in the liver as well as skeletal muscle and pancreas (30,32). Previously we have established that in the state of estrogen deficiency in both males and females, obesity is associated with an increase adipocyte volume (5) and estrogen replacement reverses the obese phenotype by causing a decrease in adipocyte volume, whereas there was very little change in adipocyte number (5,33). The present study demonstrated that E_2 replacement in males decreases obesity by reducing gonadal, visceral and BAT adipose depots. It is possible that the large adipocyte volumes observed in the estrogen deficient mice (5,33) are unable to buffer the effects of FFA and therefore the liver, another important site of triglyceride buffering (34), stores trigylcerides as a means of protecting other sites within the body. E_2 acts on the adipose depots to reduce their size and hence obviate the need for the liver to assist in the buffering of triglycerides.

The presence of central obesity is not necessarily a predictor of hepatic steatosis; as shown in estrogen deficient males and females, both are obese but only the males present with hepatic steatosis. In this case centrally mediated factors may be playing an important role; estrogen deficient males show loss of neurones in the arcuate nucleus (Arc) and medial preoptic area (MOP) regions of the hypothalamus (35). The Arc and medial eminence (ME) regions contain the highest concentration of leptin receptors in the brain (36). Leptin is a peptide hormone that is secreted from adipose tissue, which acts in a feedback fashion on its receptors that are present in the hypothalamus. Its functions are to regulate food intake and spontaneous physical activity (1) and more recently it has been shown to inhibit lipogenesis, cholesterol synthesis and stimulate

fatty acid oxidation (1,36). Leptin deficient models present with hepatic steatosis, hence there may be a reduction in leptin signalling in the ArKO males which is due to damage to the hypothalamus that is contributing to the hepatic steatosis.

Growth hormone secretion is also centrally mediated, and growth hormone deficient patients have also presented with hepatic steatosis (37,38) and reversal has been shown in one patient by growth hormone replacement (38). Growth hormone is known to act on liver to regulate the expression of certain cytochrome P450 isoforms in a sexually dimorphic fashion (39,40). Whether its action extends to triglyceride balance remains to be determined.

Previously, we have reported that estrogen deficiency in males resulted in elevated hepatic cholesterol levels, which were further increased by a high cholesterol diet (9). This present study has shown that although estrogen deficiency led to elevated hepatic cholesterol levels, E_2 replacement in the males did not reverse this phenotype. Thus hepatic cholesterol levels maybe regulated independently of obesity.

The emergence of estrogen as an important regulator of lipid homeostasis is becoming increasingly clear. This present study adds further weight to this concept and highlights the role of estrogen in regulating lipid homeostasis in males, particularly triglyceride homeostasis.

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Chapter Four: The Aromatase Knockout (ArKO) Mouse Presents with a Sexually Dimorphic Disruption to Cholesterol Homeostasis

Endocrinology 144(9):3895-3903.

Declaration for Thesis Chapter four

In the case of chapter four, contributions to the work involved the following:

Name	% contribution	Nature of contribution
Kylie Hewitt	89%	All experimental work, and preparation of manuscript
Wah Chin Boon	2%	Experimental assistance with Light cycler and editing of manuscript
Yoko Murata	2%	Experimental assistance with Light cycler
Margaret Jones	2%	Supervisor and editing of manuscript
Evan Simpson	5%	Supervisor and editing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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The Aromatase Knockout Mouse Presents with a Sexually Dimorphic Disruption to Cholesterol Homeostasis

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The aromatase knockout (ArKO) mouse cannot synthesize endogenous estrogens due to disruption of the *Cyp19* gene. We have shown previously, that ArKO mice present with age-progressive obesity and hepatic steatosis, and by 1 yr of age both male and female ArKO mice develop hypercholesterolemia. In this present study 10- to 12-wk-old ArKO mice were challenged for 90 d with high cholesterol diets. Our results show a sexually dimorphic response to estrogen deficiency in terms of cholesterol homeostasis in the liver. ArKO females presented with elevated serum cholesterol; conversely, ArKO males had elevated hepatic cholesterol levels. In response to dietary cholesterol, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase transcript levels were significantly reduced in females, whereas males showed more modest changes. Neither low density lipoprotein nor sterol regulatory element-binding protein expression levels were significantly altered by diet or genotype. The expression of *Cyp7a*, which encodes cholesterol 7α-hydroxylase, was significantly reduced in ArKO females compared with wild-type females and was increased by cholesterol feeding. *Cyp7a* expression was significantly elevated in the wild-type males on the high cholesterol diet, although no difference was seen between genotypes on the control diet. The ATP-binding cassette G5 and ATP-binding cassette G8 transporters do not appear to be regulated by estrogen. The expression of acyl-coenzyme A:cholesterol acyltransferase 2 showed a sexually dimorphic response, where estrogen appeared to have a stimulatory effect in females, but not males. This study reveals a sexually dimorphic difference in mouse hepatic cholesterol homeostasis and roles for estrogen in the regulation of cholesterol uptake, biosynthesis, and catabolism in the female, but not in the male. (*Endocrinology* 144: 3895–3903, 2003)

THE POSSIBILITY that estrogen may play an important role in regulating cholesterol homeostasis has been suggested based on studies showing that premenopausal women have a lower risk of cardiovascular disease than male age-matched controls; however, postmenopausally, when circulating estrogen levels are reduced, their risk rises compared with premenopausal women (1–3). Elevated serum low-density lipoprotein (LDL) levels have been associated with an increased risk of cardiovascular disease (4), whereas high-density lipoprotein (HDL) levels have been shown to have the reverse effect (5). Oral administration of estrogen to postmenopausal women results in lowered levels of LDL (4). This evidence suggests that estrogen plays an important role in cholesterol homeostasis and is protective in terms of cholesterol-associated pathologies.

Models of estrogen deficiency have been used to gain insight into the mechanisms of this regulation. These models are the aromatase knockout (ArKO) mouse (6), the estrogen receptor α knockout mouse (7), the estrogen receptor β knockout mouse (8), and the double estrogen receptor knockout mouse (9). ArKO mice presented with age-progressive obesity and hepatic steatosis. By 1 yr of age, both male and female ArKO mice developed hypercholesterolemia, and male ArKO mice exhibited elevated triglycerides (10). Estrogen receptor α knockout and double estrogen receptor knockout mice presented with a similar phenotype as the ArKOs (11, 12), whereas no lipid phenotype was described in estrogen receptor β knockout mice (12). These results indicate that in the absence of estrogen there is a disruption of lipid homeostasis, and presumably this is acting primarily through ERα. In addition to these mouse knockout models, three adult men have been reported with aromatase deficiency (13–16), and one adult male with a defect in ERα has been described by Smith *et al.* (17). These men showed impaired glucose and lipid metabolism (18), and at least one of the aromatase-deficient patients presented with hepatic steatosis (16, 18).

It is generally recognized that cholesterol homeostasis is a tightly regulated process, as excess circulating cholesterol is associated with increased risk of cardiovascular disease (19). Cholesterol homeostasis is mainly achieved by regulation of transcription of the enzymes involved in cholesterol synthesis, uptake, and clearance. When sterols in cells are low, the NH_2-terminal domain of sterol regulatory element-binding protein (SREBP)2 is cleaved so that it can translocate from the endoplasmic reticulum to the nucleus and up-regulate the transcription of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase and HMG CoA synthase, enzymes

Abbreviations: ABCG5, ATP-binding cassette G5; ABCG8, ATP-binding cassette G8; ACAT2, acyl-coenzyme A:cholesterol acyltransferase 2; ArKO, aromatase knockout; HDL, high-density lipoprotein; HMG CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A; LDL, low-density lipoprotein; LDLR, LDL receptor; LXR, liver X receptor; SREBP, sterol regulatory element-binding protein; WT, wild-type.

involved in the *de novo* synthesis of cholesterol. The LDL receptor (LDLR) is also regulated through this process to allow the uptake of cholesterol from serum (19). Cholesterol is cleared from the body via the liver through the bile acid pathway and also by direct secretion into the bile. The enzyme cholesterol 7α-hydroxylase, encoded by the *Cyp7a* gene, catalyzes the rate-limiting step in the pathway of bile acid synthesis and is positively regulated by cholesterol. This is believed to be mediated, at least in rodents, by the formation of oxygenated cholesterol metabolites that serve as ligands for liver X receptor (LXR)α, an orphan member of the nuclear receptor superfamily that is required for *Cyp7a* transcriptional activity (20). Similarly, the ATP-binding cassette G5 (ABCG5) and ATP-binding cassette G8 (ABCG8) transporters, responsible for clearing cholesterol from the liver, are also regulated by LXRα (21).

In the present study the role of estrogen in regulating cholesterol homeostasis by the liver has been examined using the ArKO mouse model (6). The aim of the study was to gain further insight into the role of estrogen to regulate cholesterol homeostasis. To achieve this, ArKO mice were challenged with high cholesterol diets, and transcripts of enzymes and factors involved in cholesterol synthesis, uptake, and clearance were measured.

Materials and Methods

Mice

The ArKO mice were generated by deleting 90% of exon 9 of the *Cyp19* gene as described by Fisher *et al.* (6). Wild-type (WT) and homozygous null offspring were generated by heterozygous matings. The genotype of the offspring was determined by PCR as described by Robertson *et al.* (22). Experimental design and animal usage were approved by the Monash Medical Center animal ethics committee. The animals were housed in specific pathogen-free conditions and had unlimited access to drinking water and food.

Diets

Soy-free mouse chow (Glen Forest Stock Feeders, Perth, Australia) was the control diet used to feed the mice; it contains wheat meal instead of the soy meal found in regular mouse chow, as isoflavones in soy are known to have estrogenic effects (23). This diet contains 15% of calories as fat (0.02% cholesterol), 20% calories as protein, and 65% of calories as carbohydrate. Intermediate and high cholesterol diets were fed to the mice to challenge their lipid homeostasis. The intermediate diet had 0.2% cholesterol added to the soy-free mouse chow; this is 2-fold more than what is normally needed to maintain homeostasis. The high cholesterol diet had 2% cholesterol added to the soy-free mouse chow, which is 20- to 30-fold more than that normally needed to maintain homeostasis. ArKO and WT males and females were fed the control diet (0% added cholesterol to a soy-free diet), the 0.2% cholesterol diet, or the 2% cholesterol diet for 90 d beginning at 10–12 wk of age.

Tissue collection

Mice were killed by cervical dislocation. Truncal blood was collected after decapitation. Blood was allowed to clot, and serum was collected and stored at −20 C. The liver was removed, weighed, snap-frozen in liquid nitrogen, and stored at −80 C for gene and lipid analyses.

Measurement of serum and hepatic lipids

Cholesterol and HDL were quantified in the bloodstream using Cholesterol Flex and automated HDL cholesterol kits, respectively (Dade Behring, Newark, DE). Hepatic cholesterol levels were quantified after homogenization of 0.2 g liver in 10 ml chloroform/methanol (2:1, vol/vol) (20). Samples were centrifuged for 20 min at 800 × *g*; the lipid phase was removed, and chloroform was evaporated off. Total cholesterol was quantified using the Cholesterol 20 kit (352-20, Sigma-Aldrich Corp., St. Louis, MO).

Gene analysis

RNA was extracted from the liver using the phenol-chloroform method (Ultraspec RNA, Fisher Biotech, Australia) and was quantified spectrophotometrically. Two-step RT-PCR was performed using random primers (Roche, Mannheim, Germany) and AMV reverse transcriptase enzyme (Promega, Madison, WI). A LightCycler (Roche) was used to quantitate mouse transcripts using specific primer pairs. Primer pairs were shown to be specific through a single peak in the melting curves, and a single product was seen on an ethidium bromide (Sigma-Aldrich Corp.) agarose (Promega) gel corresponding to the appropriate product size as measured by a 1-kb ladder (Promega). To further confirm the primer specificity, PCR products were sequenced to confirm their identities. Primer sequences are shown in Table 1.

All samples were normalized to 18S. All samples were run individually in three separate RT reactions, transcripts were measured using real-time PCR, and then the data were presented as a mean of the three

TABLE 1. Primer sequences and product size

Gene	Primer pairs	Product size (bp)
HMG CoA reductase	F: 5′-GTGGGACCAACCTTCTACCTCA-3′ R: 5′-ACTGAACTGAAGCGCGGGCAT-3′	275
LDLR	F: 5′-GTGGAGGAACTGGCGGCTGAAG-3′ R: 5′-CTCCAGACCTCCCCATCCAGCAC-3′	248
SREBP2	F: 5′-CACAATATCATTGAAAAGCGCTACCGGTCC-3′ R: 5′-TTTTTCTGATTGGCCAGCTTCAGCACCATG-3′	200 (47)
Cholesterol 7α-hydroxylase	F: 5′-TCTGGGGGATTGCTGTGGTAGT-3′ R: 5′-GTCCACTTCATCACAAACTCCCTG-3′	230
ABCG5 transporter	F: 5′-CTGCTGAGGCGAGTAACAAGAAAC-3′ R: 5′-GTCCTCCCCTTCAGCGTCATCG-3′	322
ABCG8 transporter	F: 5′-GACCTGCCCACGCTGCTCATTCAT-3′ R: 5′-CCGCAGGTTTGTCAGCCAGTAGAT-3′	330
ACAT2	F: 5′-GAGACAYACCCCAGGACACC-3′ R: 5′-GTTGGCAAAGACAGGGACAC-3′	133
18S	F: 5′-CGG CTA CCA CAT CCA AGG AA-3′ R: 5′-GCT GGAATT ACCGCGGCT-3′	180

F, Forward; R, reverse.

consistent runs. Interassay variability was assessed using the same standards in repeated runs and assessing the crossing points to ensure consistency between runs.

Statistical analysis

All graphs were expressed as the mean ± SEM. Univariate ANOVA was used to determine overall statistical differences. Genotypes within a diet were compared using univariate ANOVA. When there were three diets, Tukey's *post hoc* test was used to determine significance (SPSS version 10.0 for Windows, SPSS, Inc., Chicago, IL).

Results

Serum cholesterol levels

Serum cholesterol and HDL were measured in males and females. Overall, the female ArKO mice had significantly elevated levels of female serum cholesterol and HDL compared with WT animals ($P = 0.015$; $F_1 = 6.716$ and $P = 0.017$; $F_1 = 6.427$, respectively). For individual diets, the ArKO females on the control diet had significantly elevated levels of serum cholesterol compared with WT controls ($P = 0.019$; $F_1 = 6.736$; Fig. 1A), and serum HDL levels were significantly elevated in ArKO females on the control diet compared with control WT mice ($P = 0.006$; $F_1 = 12.614$; Fig. 1C). No significant changes were seen in serum cholesterol or HDL between ArKO or WT mice on the 0.2% ($P = 0.293$; $F_1 = 1.245$ and $P = 0.455$; $F_1 = 0.609$, respectively) or the 2% cholesterol diet ($P = 0.310$; $F_1 = 1.157$ and $P = 0.291$; $F_1 = 1.261$). There was no significant difference between diets for either serum cholesterol ($P = 0.192$; $F_2 = 1.754$) or HDL ($P = 0.602$; $F_2 = 0.517$).

Conversely, males on the control diet showed no difference between ArKO and WT in serum cholesterol levels ($P = 0.117$; $F_1 = 3.005$) or either the 0.2% or 2% cholesterol diets ($P = 0.585$; $F_1 = 0.318$ and $P = 0.730$; $F_1 = 0.158$, respectively; Fig. 1B). When they were fed the 2% cholesterol diet, there was a significant reduction in serum cholesterol levels for both genotypes compared with animals on the control diet ($P = 0.012$; $F_2 = 5.562$; Fig. 1B). There was also a reduction in serum cholesterol levels in the males fed the 0.2% cholesterol diet compared with animals fed the control diet, ($P = 0.056$; $F_2 = 5.562$), although it did not reach significance. Similarly, serum HDL levels did not differ between genotypes on the control diet ($P = 0.429$; $F_1 = 0.686$), the 0.2% cholesterol diet ($P = 0.130$; $F_1 = 2.717$), or the 2% cholesterol diet ($P = 0.900$; $F_1 = 0.017$). However, when they were fed the 2% cholesterol diet, there was a significant reduction in HDL levels compared with control-fed animals ($P = 0.005$; $F_2 = 6.353$; Fig. 1D). There was also was a reduction in serum HDL levels in males fed the 0.2% cholesterol diet compared with animals fed the control diet ($P = 0.069$; $F_2 = 6.353$).

Hepatic cholesterol levels

Hepatic cholesterol levels were measured in both males and females. Female ArKO mice on the control diet showed significantly lower levels of hepatic cholesterol compared with WT ($P = 0.05$; $F_1 = 4.217$; Fig. 2A). Hepatic cholesterol levels were elevated significantly in ArKO and WT females fed both the 0.2% and 2% cholesterol diets compared with control-fed animals ($P = 0.000$ and $P = 0.000$, respectively; $F_2 = 19.239$; Fig. 2A). No differences were seen between genotypes on the 0.2% and 2% cholesterol diets ($P = 0.251$; $F_1 = 1.507$ and $P = 0.275$; $F_1 = 1.405$, respectively). The male mice showed the opposite effect, namely that ArKO mice on the control diet had significantly elevated levels of hepatic cholesterol compared with WT controls ($P = 0.000$; $F_1 = 217.187$; Fig. 2B). When the male mice were fed the 0.2% and 2% cholesterol diets, there was a significant increase in hepatic cholesterol levels in all groups compared with controls ($P = 0.000$ and $P = 0.000$, respectively; $F_2 = 47.439$; Fig. 2B).

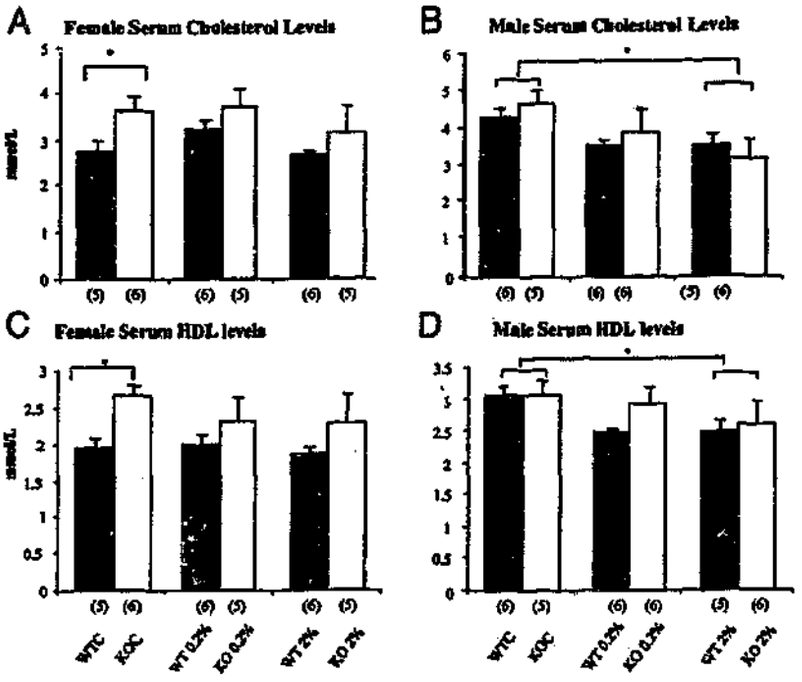

FIG. 1. Serum lipid profiles. A, Female serum cholesterol levels. Overall, ArKOs have significantly elevated serum cholesterol levels compared with WT animals ($P = 0.015$; $F_1 = 6.716$). ArKO controls have significantly elevated serum cholesterol levels compared with WT controls ($P = 0.019$; $F_1 = 6.736$). B, Male serum cholesterol levels. Levels from males on the control diet were significantly elevated compared with males fed the 2% cholesterol diet ($P = 0.012$; $F_2 = 5.562$). C, Female serum HDL cholesterol levels. Overall, ArKOs have significantly elevated serum HDL levels compared with WTs ($P = 0.017$; $F_1 = 6.427$). ArKO controls have significantly elevated serum HDL levels compared with WT controls ($P = 0.006$; $F_1 = 12.614$). D, Male serum HDL cholesterol levels. Males on the control diet have significantly elevated levels compared with males on the 2% cholesterol diet ($P = 0.005$; $F_1 = 6.353$). ArKO (□) and WT (■) mice. wtc and koc, WT and ArKO on the control diet; WT 0.2% and KO 0.2%, WT and ArKO on the 0.2% cholesterol diet; WT 2% and KO 2%, WT and ArKO on the 2% cholesterol diet. *, $P < 0.05$.

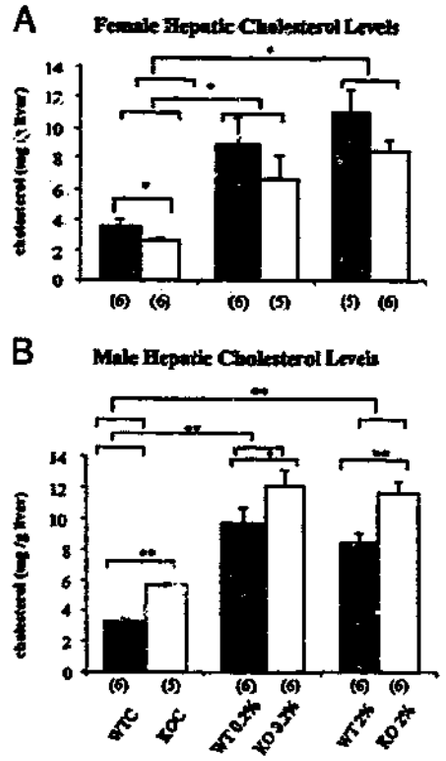

FIG. 2. Hepatic cholesterol levels. A, Female hepatic cholesterol levels. ArKO mice on the control diet have significantly lower levels of hepatic cholesterol compared with WT ($P = 0.05$; $F_1 = 4.217$). Both genotypes fed the 0.2% and 2% cholesterol diets have significantly higher levels of hepatic cholesterol compared control fed animals ($P = 0.000$; $F_2 = 19.239$). B, Male hepatic cholesterol levels. ArKO compared with WT on the control diet had significantly elevated hepatic cholesterol ($P = 0.000$; $F_1 = 217.187$). ArKO mice fed the 0.2% and 2% cholesterol diets have significantly higher levels of hepatic cholesterol compared with diet-matched controls (for both, $P = 0.000$; $F_2 = 47.439$). Between genotypes there was a significant difference for both 0.2% and 2% cholesterol diets ($P = 0.030$; $F_1 = 6.676$ and $P = 0.003$; $F_1 = 15.48$, respectively). □, ArKO mice; ■, WT mice. WTC and KOC, WT and ArKO on the control diet; WT 0.2% and KO 0.2%, WT and ArKO on 0.2% cholesterol diet; WT 2% and KO 2%, WT and ArKO on 2% cholesterol diet. *, $P < 0.05$; **, $P < 0.01$.

However, the hepatic cholesterol content of male ArKOs on the 0.2% and 2% cholesterol diets remained significantly elevated compared with their diet-matched controls ($P = 0.030$; $F_1 = 6.676$ and $P = 0.003$; $F_1 = 15.48$, respectively; Fig. 2B).

Expression of genes involved in de novo cholesterol synthesis and uptake

To gain an understanding of the mechanisms that led to the altered cholesterol homeostasis, real-time PCR was performed to quantitate the expression of genes involved in cholesterol metabolism. Transcripts were measured in ArKO and WT mice on the control and 2% cholesterol diets. Female ArKO mice on the control diet showed no statistically significant change in HMG CoA reductase transcript levels compared with WT controls ($P = 0.269$; $F_1 = 1.368$) or when they fed 2% cholesterol ($P = 0.568$; $F_1 = 0.348$; Fig. 3A). When they were fed the 2% cholesterol diet, there was a significant reduction in transcript levels for both ArKO and WT females ($P = 0.017$; $F_1 = 6.766$; Fig. 3A). For the males, however, there were no differences between genotype on either diet (control diet: $P = 1.00$; $F_1 = 0.000$; 2% cholesterol diet: $P = 0.212$; $F_1 = 1.778$) or between diets ($P = 0.130$; $F_1 = 2.501$; Fig. 3B).

The LDLR is responsible for the uptake of LDL cholesterol from serum. No changes were seen for males and females regardless of genotype (LDLR control diet: males, $P = 0.477$; $F_1 = 0.552$; females, $P = 0.774$; $F_1 = 0.087$; 2% cholesterol diet: males, $P = 0.309$; $F_1 = 1.148$; females, $P = 0.365$; $F_1 = 0.900$) or between diets (females, $P = 0.093$; $F_1 = 3.110$; males, $P = 0.961$; $F_1 = 0.002$; Fig. 3, C and D). SREBP2 is responsible for the transcriptional regulation of both HMG CoA reductase and the LDLR. Its transcripts showed no changes in expression levels in males and females regardless of genotype (control diet: females, $P = 0.248$; $F_1 = 1.056$; males, $P = 0.217$; $F_1 = 1.763$; 2% cholesterol diet: females, $P = 0.246$; $F_1 = 1.518$; males, $P = 0.416$; $F_1 = 0.720$) or between diets (females, $P = 0.094$; $F_1 = 3.097$; males, $P = 0.822$; $F_1 = 0.052$; Fig. 3, E and F).

Expression of genes involved in the clearance of cholesterol

Cholesterol 7α-hydroxylase catalyzes the rate-limiting step of cholesterol conversion into bile acids and is encoded by the *Cyp7a* gene. Female ArKO mice had significantly lower levels of expression of *Cyp7a* compared with WT controls ($P = 0.044$; $F_1 = 4.616$; Fig. 4A). When the female mice were fed the 2% cholesterol diet, there was a significant elevation in *Cyp7a* transcript levels in ArKO females compared with ArKO controls ($P = 0.049$; $F_1 = 4.414$; Fig. 4A). *Cyp7a* expression was lower in male livers compared with females and was not different in male ArKO and WT on the control diet ($P = 0.726$; $F_1 = 0.130$) *vs.* when they were fed the 2% cholesterol diet ($P = 0.212$; $F_1 = 1.778$). However, 2% cholesterol up-regulated *Cyp7a* expression in WT males, but not in ArKO males ($P = 0.025$; $F_1 = 6.529$; Fig. 4B).

The role of the ABCG5 and ABCG8 transporters is to remove excess cholesterol from both liver and intestine. Females on the control diet had no difference in both transporter transcript levels between genotypes (ABCG5: $P = 0.506$; $F_1 = 0.476$; ABCG8: $P = 0.631$; $F_1 = 0.245$) or when fed the 2% cholesterol diet (ABCG5: $P = 0.728$; $F_1 = 0.128$; ABCG8: $P = 0.932$; $F_1 = 0.008$; Fig. 4, C and E). Challenge with the high cholesterol diet resulted in a significant up-regulation in both genotypes compared with controls ($P = 0.009$; $F_1 = 8.514$ and $P = 0.002$; $F_1 = 12.135$, respectively; Fig. 4, E and C). Similarly, males on the control diet also showed no differences in transcript levels for ABCG5 and ABCG8 transporters ($P = 0.205$; $F_1 = 1.844$ and $P = 0.231$; $F_1 = 1.653$, respectively) or on the 2% cholesterol diet (ABCG5: $P = 0.205$; $F_1 = 1.844$; ABCG8: $P = 0.651$; $F_1 = 0.218$; Fig. 4, D and F). However, when the diet was supplemented with 2% cholesterol, there was a significant up-regulation in expression only for the ABCG8 transporter in both ArKO and WT ($P = 0.022$; $F_1 = 6.209$); ABCG5 transporter expression did not change ($P = 0.822$; $F_1 = 0.052$; Fig. 4, D and F).

Acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) catalyzes the formation of cholesterol esters from unesterified cholesterol in the liver. Transcript levels were significantly reduced in female ArKO control mice compared with

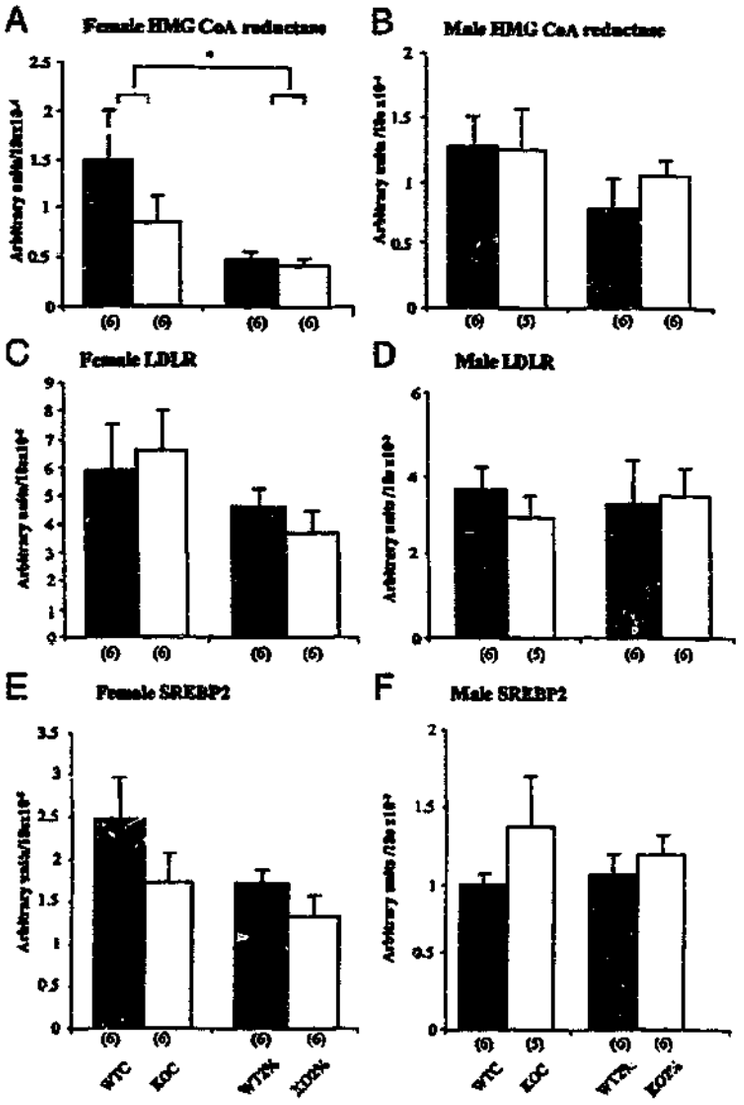

FIG. 3. Levels of transcripts for genes regulating *de novo* cholesterol synthesis and uptake. A, Female HMG CoA reductase transcript levels. Females on the control diet have significantly higher levels of transcripts for HMG CoA reductase compared with 2% cholesterol-fed females ($P = 0.017$; $F_1 = 6.766$). B, Male HMG CoA reductase transcript levels. P = NS, changes between genotypes for either diet or between diets. C, Female LDLR transcript levels. P = NS, between genotypes for either diet or between diets. D, Male LDLR transcript levels. P = NS, between genotypes for either diet or between diets. C, Female SREBP2 transcript levels. P = NS, between genotypes for either diet, or between diets. F, Male SREBP2 transcript levels. P = NS, between genotypes for either diet or between diets. □, ArKO mice; ■, WT mice. WTC and KOC, WT and ArKO on the control diet; WT 2% and KO 2%, WT and ArKO on the 2% cholesterol diet.

WT ($P = 0.015$; $F_1 = 7.146$), No significant changes were seen between genotypes for the 2% cholesterol diet ($P = 0.123$; $F_1 = 2.836$; Fig. 4G). No significant changes were seen in ACAT2 levels for 2% cholesterol-fed animals ($P = 0.123$; $F_1 = 2.836$). ACAT2 expression was not different between male ArKC and WT on the control diet ($P = 0.246$; $F_1 = 1.540$) or the 2% cholesterol diet ($P = 0.187$; $F_1 = 2.011$. Both genotypes, however, responded with a significant up-regulation of ACAT2 when fed the 2% cholesterol diet ($P = 0.04$; $F_1 = 11.014$; Fig. 4H).

Discussion

The rodent liver has long been known to have gender-specific properties, for example, the sexually dimorphic expression of certain members of the P450 superfamily involved in the metabolism of steroid hormones (24, 25). This difference has been related to the differing patterns of GH secretion in males and females. We have previously reported that male ArKO mice are more prone to the development of hepatic steatosis than are female ArKO mice (26). Herein we report on a sexually dimorphic regulation of cholesterol homeostasis as revealed by the ArKO phenotype. The results are summarized in Table 2 and indicate a role for estrogen in the regulation of cholesterol metabolism by the liver of female, but not male, mice. On the other hand, the livers of both sexes responded to a high cholesterol diet in a broadly similar fashion, although there were some differences in the details.

Female hepatic phenotype

We observed that although cholesterol feeding did not result in a rise in serum cholesterol levels in female WT mice, there was a 3-fold increase in hepatic cholesterol levels. This was accompanied by a 3-fold decrease in the levels of transcripts for HMG CoA reductase and more modest declines in the levels of transcripts for the LDLR and SREBP2 (these did not reach statistical significance). These results are consistent with the concept that dietary cholesterol enters the

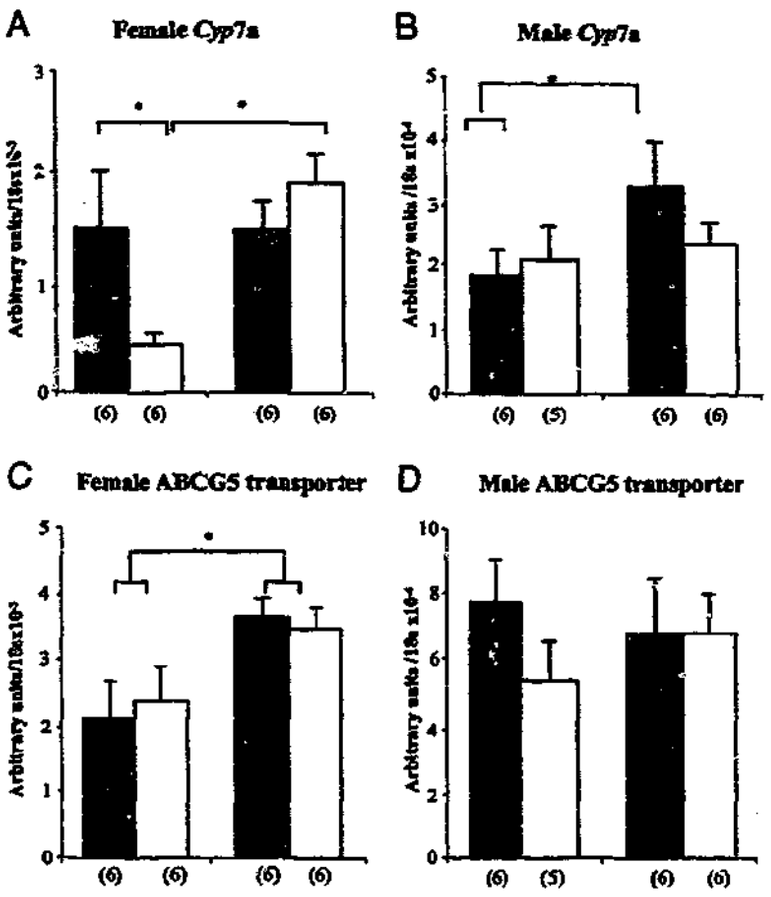

FIG. 4. Levels of transcripts for genes regulating cholesterol clearance. A, Female Cyp7a transcript levels. ArKO controls have significantly lower levels of Cyp7a compared with control WT ($P = 0.044$; $F_1 = 4.616$). ArKO females fed the 2% cholesterol diet have significantly elevated levels of Cyp7a compared with ArKO control-fed females ($P = 0.049$; $F_1 = 4.414$). B, Male Cyp7a transcript levels. A significant increase in Cyp7a was seen for WT fed 2% cholesterol compared with control animals ($P = 0.025$; $F_1 = 6.529$). C, Female ABCG5 transporter transcript levels. Control-fed females had significantly lower levels of ABCG5 transporter compared with 2% cholesterol-fed females ($P = 0.009$; $F_1 = 8.514$). D, Male ABCG5 transporter transcript levels. P = NS, differences were seen between genotypes for either diet or between diets. E, Female ABCG8 transporter transcript levels. Control-fed females has significantly lower levels of ABCG8 transporter transcript levels compared with 2% cholesterol-fed females ($P = 0.002$; $F_1 = 12.135$). F, Male ABCG8 transporter transcript levels. Control-fed males had significantly lower levels of ABCG8 transporter transcript levels compared with 2% cholesterol fed males ($P = 0.022$; $F_1 = 6.209$). G, Female ACAT2 transcript levels. Control-fed ArKOs had significantly lower levels of ACAT2 compared with WT controls ($P = 0.015$; $F_1 = 7.146$). H, Male ACAT2 transcript levels. Control-fed males had significantly lower levels of ACAT2 compared with 2% cholesterol-fed males ($P = 0.004$; $F_1 = 11.014$). ArKO (□) and WT (■) mice. WTC and KOC, WT and ArKO on the control diet; WT 2% and KO 2%, WT and ArKO on the 2% cholesterol diet. *, $P < 0.05$.

bloodstream in the form of chylomicrons, which are metabolized by peripheral lipoprotein lipase to remove much of the triglyceride component. The resulting cholesterol-enriched remnants are then cleared by the liver (27). This cholesterol entering the liver would then serve to inhibit the *de novo* synthesis of cholesterol and its uptake by the LDLR, at least in part by inhibiting the expression of the genes encoding these protein (19, 28). Such inhibition is believed to be mediated primarily by oxysterols formed from the hepatic cholesterol acting to inhibit the cleavage of SREBP2 to form the N-terminal fragment released from the endoplasmic reticulum. This enters the nucleus to act as a transcription factor for the genes encoding HMG CoA reductase and the LDLR (19). In addition, cholesterol has been shown to stimulate the transcription of Cyp7a, the gene encoding cholesterol 7α-hydroxylase, the rate-limiting step in bile acid synthesis. This is believed to be mediated by oxysterols acting as ligands for LXRα (20, 29). Nevertheless, in the present study Cyp7a transcript levels were not increased in the wild-type mice upon feeding cholesterol.

In the case of ArKO mice on the regular soy-free diet, serum cholesterol levels were elevated, and liver cholesterol was decreased relative to the WT mice. This is suggestive of a defect in cholesterol clearance from the blood by the livers of the ArKO mice. There was a concomitant decrease in transcript levels for HMG CoA reductase relative to WT, but little or no change in the levels of transcripts for the LDL receptor or SREBP2. The most dramatic change was a 3-fold decrease in transcript levels for Cyp7a. Several studies have examined the role of estrogens in the regulation of HMG CoA reductase transcripts and protein with variable results (30–33). The promoter of HMG CoA reductase has an estrogen-responsive element-like sequence, RED-ERE (34). Studies to date are unclear on whether estrogen acts on this *in vivo*. HMG CoA reductase activity has been shown to be responsive to estradiol (10 nM) in MCF7 cells and was strongly inhibited by the antiestrogen ICI 164,384. However, in this study there were no changes in transcript levels (34). A study in intact female rats showed a biphasic effect of estrogen. Whereas physiological levels of estrogen led to an increase in HMG CoA reductase activity, higher levels of estrogen (1 mg/kg·d) reduced HMG CoA reductase activity back to control levels. These studies indicated that if estrogen does play a role in regulating cholesterol synthesis through the regulation of HMG CoA reductase, it appears to be acting at the level of activity rather than transcription. It may be, therefore, that the lower levels of hepatic cholesterol in the ArKO females compared with controls are due to a down-regulation of HMG CoA reductase activity.

On the other hand, several studies have shown that estrogen up-regulates cholesterol 7α-hydroxylase (35–38); thus, the lack of estrogen action on the livers of the ArKO females together with the lower hepatic cholesterol levels may be the reason for the significant decrease in Cyp7a

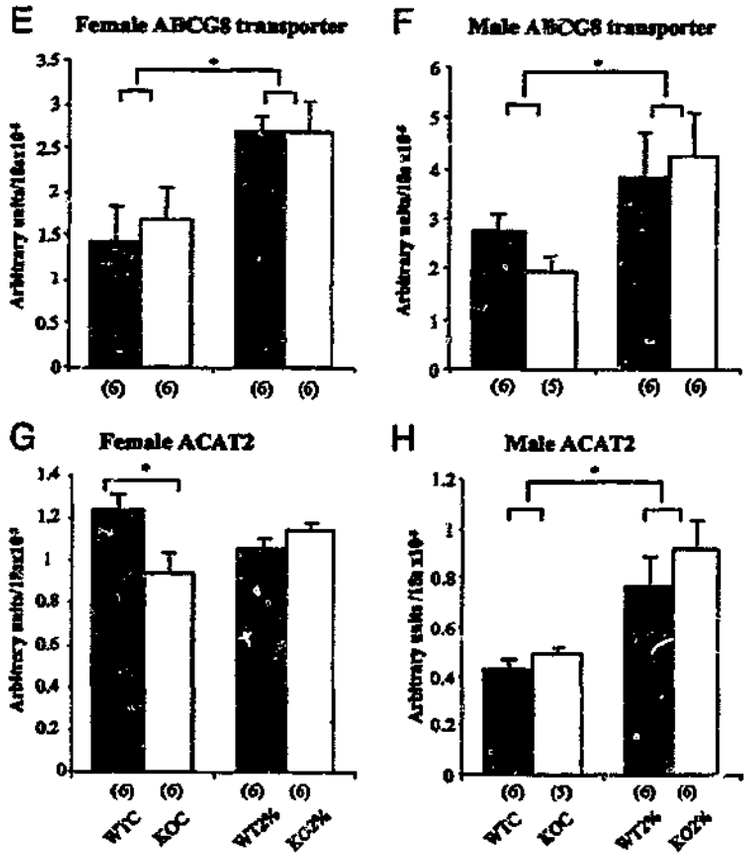

FIG. 4. *Continued*

TABLE 2. Summary of results

Parameters measured	Absence of estrogen		2% Cholesterol diet			
			F		M	
	F	M	KO	WT	KO	WT
Serum cholesterol	⇑	⇔	⇔	⇔	⇓	⇓
Serum HDL	⇑	⇔	⇔	⇔	⇓	⇓
Hepatic cholesterol	⇓	⇑	⇑	⇑	⇑	⇑
HMG CoA reductase	⇔	⇔	⇓	⇓	⇔	⇔
LDLR	⇔	⇔	⇔	⇔	⇔	⇔
SREBP2	⇔	⇔	⇔	⇔	⇔	⇔
Cholesterol 7α-hydroxylase	⇓	⇔	⇑	⇔	⇔	⇑
ABCG5 transporter	⇔	⇔	⇑	⇑	⇔	⇔
ABCG8 transporter	⇔	⇔	⇑	⇑	⇑	⇑
ACAT2	⇓	⇔	⇔	⇔	⇑	⇑

Column 1 refers to parameters measure; *column 2* refers to ArKO compared to WT on the control diet; *column 3* refers to 2% cholesterol diet compared to control diet. F, Female; M, male; KO, ArKO. ⇑, Increase; ⇓, decrease; ⇔, no change.

transcript levels that we observed. Thus, the failure of cholesterol feeding to increase Cyp7a transcripts in the WT liver may be due to the fact that the gene is already activated by estrogen.

Consistent with these concepts, cholesterol feeding of the ArKO females resulted in an increase in liver cholesterol to a lesser extent than in WT animals, but a decline in HMG CoA reductase transcripts to the levels seen in both ArKO and WT animals fed cholesterol. There were no changes in the levels of transcripts for the LDLR and SREBP2. However, most dramatically there was a 4-fold increase in the expression of Cyp7a transcript levels upon feeding cholesterol to ArKO females to levels similar to those seen in WT animals. We conclude from these studies that in the livers of female mice, estrogen and cholesterol induce the expression of Cyp7a to a similar extent, but the effects are not additive. The lack of change in LDLR transcript levels in ArKO animals compared with WT is perhaps surprising in the light of reports that estrogens increase the levels of LDLR and its mRNA (39–41) and also the high circulating cholesterol levels present in the absence of estrogen. However, these studies generally employed pharmacological levels of 17α-ethinyl estradiol, and so it is unclear whether physiological levels of estradiol have the capacity to regulate LDLRs. It is also important to note that inhibition of estrogen action with compounds such as tamoxifen and clomiphene (60 mg/kg) did not decrease LDLR expression (30). Thus, although estrogen at high concentrations is a potent stimulator of the LDLR it may not be required for normal functioning of the receptor.

Regarding the ABCG cholesterol transporters, it appears that neither the ABCG5 nor the ABCG8 transporter was affected by the estrogenic state of the mice, although both were induced by cholesterol feeding. This is consistent with the role of LXRα to regulate the expression of these transporters (21). In the female liver, ACAT2 transcript levels were suppressed in the absence of estrogen; this may indicate a role for estrogen in ACAT2 regulation, or it may possibly be due to lower levels of hepatic

cholesterol observed in the ArKO females. Cholesterol feeding raised the levels of ACAT2 expression in the ArKO females to a value not different from WT.

Male hepatic phenotype

Overall the levels of the various transcripts in the male livers compared with those of the females would suggest a similar responsiveness to cholesterol feeding, but a failure to respond to estrogen. Thus, the cholesterol content of the male livers was increased upon cholesterol feeding, and this was actually accompanied by a decrease in circulating levels in contrast to those in females. There was also an increase in liver cholesterol in ArKO males, in contrast to a decrease seen in ArKO females, suggesting that the inhibitory effect of estrogen deprivation on cholesterol uptake by the female livers was not present in the males, but that elevated androgens might stimulate cholesterol uptake by the liver. Furthermore, the level of transcripts for Cyp7a increased upon cholesterol feeding in the WT males, but was not affected by the estrogenic state of the animals, again in contrast to the females where the level of Cyp7a was dramatically decreased in the ArKO livers compared with those in WT mice.

In the livers of male mice, the ABCG8 transporter transcripts behaved similarly to those in the females; namely, a stimulation upon cholesterol feeding, but no effect of estrogenic status. However, the ABCG5 transporter was unresponsive, and the WT levels were 2- to 3-fold less compared with those in females. In the case of ACAT2, this did respond to cholesterol feeding with a 3-fold elevation in transcript levels. This was in contrast to the female liver, where wild-type ACAT2 transcript levels were elevated 2-fold compared with those in the male. Estrogen did not appear to affect ACAT2 expression in the males, in which the absence of estrogen led to lower transcript levels in the females.

An important question that arises is the origin of the estrogen that would influence the livers of WT animals. Estrogen levels in WT males are undetectable in the peripheral circulation, yet the male ArKO liver displays marked hepatic steatosis (26). An interesting potential source of estrogen that would affect the livers of both male and female mice is the gastric mucosa. Recently Ueyama *et al.* (42) showed that gastric parietal cells were a potent site of aromatase activity, which resulted in high circulating estradiol levels in the hepatic portal vein, but not in the peripheral circulation, indicating that estradiol was cleared by the liver. Aromatase activity in gastric mucosa appeared to be roughly equal in males and females. This, then, would provide a nonsexually dimorphic source of estrogen to the liver. It may be assumed, therefore, that the differences between the livers of male and female mice with regard to the effects of estrogen on cholesterol metabolism must reflect differences in the responsiveness of the livers of males and females to the presence of estrogen. Whether androgens play a role in this differential responsiveness remains to be ascertained. Alternatively, and perhaps additionally, the action of estrogen on the liver may be secondary to action in the brain as a consequence of local aromatase activity in the brain. As mentioned previously, sexually dimorphic differences in the levels of certain hepatic cytochrome P450 levels have been attributed to different patterns of GH secretion in males and females (43–45). Resolution of this issue must await the generation of a mouse with a brain-specific inactivation of the aromatase gene.

In conclusion, we have demonstrated a role for estrogen in the regulation of cholesterol metabolism by the livers of female, but not male mice, indicating a sexually dimorphic response in this important homeostatic pathway.

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Chapter Five: General Discussion and Conclusions

5.1 Summary

Previously epidemiological studies of postmenopausal women have suggested that estrogen may play a role in regulating cholesterol and triglyceride homeostasis. The work described in this thesis has examined the effects of estrogen deficiency in both male and female mice on hepatic triglyceride and cholesterol homeostasis. Overall these studies have revealed sexual dimorphism in the regulation of these pathways in the absence of estrogen, namely that males appear to have a more dramatic phenotype in the absence of estrogen in terms of triglyceride homeostasis. Conversely, examination of cholesterol homeostasis revealed alterations in both estrogen deficient males and females, however this disruption differs between the genders. Therefore these studies reveal that estrogen is required for normal lipid homeostasis in both males and females, but the mechanisms by which estrogen regulates homeostasis differs. This is particularly important finding in terms of searching for suitable drugs to treat lipid abnormalities in both sexes.

After cholesterol was added to the diets of the mice to challenge cholesterol homeostasis, the hepatic steatosis present in estrogen deficient males was reversed and serum triglyceride levels in estrogen deficient females were lowered. Increased dietary cholesterol also lowered serum cholesterol levels in the estrogen deficient males, however the mechanisms are not completely understood.

5.2 Triglyceride homeostasis

Estrogen deficiency in male mice leads to hepatic steatosis due to an accumulation of hepatic triglycerides (described in chapter two). To elucidate the mechanism that lead

to this disruption, gene expression of key enzymes involved in triglyceride homeostasis was measured using real time PCR. These data revealed increased lipogenesis as indicated by increases in FAS and ACCα expression and an increase in FA transport as seen by elevated ADRP expression. Due to the elevated level of hepatic triglycerides one might expect a compensatory increase in VLDL production for export of triglycerides from the liver. However, the measurement of apoE expression revealed no change, possibly indicating that there was no increased output, thereby exacerbating the phenotype. We did not however, see any alterations in FA β-oxidation unlike one other group with an ArKO mouse model, where they showed it was decreased. Surprisingly estrogen deficient females did not display any alterations in hepatic triglyceride homeostasis. The only disruption the ArKO female mice displayed was elevated levels of serum triglycerides.

The incidence of obesity is approaching epidemic proportions in the western world and a consequence of this condition is hepatic steatosis. Central obesity, which is what is commonly associated with estrogen deficiency, rather than peripheral obesity, is related to the condition IR or the 'metabolic syndrome'. Previously, we have shown that estrogen deficiency leads to obesity in both genders (Jones *et al.* 2000). This obesity is due to increased adipocyte volume (Jones *et al.* 2000), which is a consequence of increased filling of the adipocyte. Gene expression studies revealed increased LPL expression in adipose tissue (Misso *et al.* 2003b), which catalyses hydrolysis of serum triglycerides releasing FFA and sn2-monoglycerides for uptake into the adipose tissue (Fredrikson & Belfrage 1983). Therefore estrogen deficiency appears to lead to increased uptake of FFA into adipose tissue. Release of FFA from adipocytes is dependent on HSL. It catalyses hydrolysis of intracellular triglycerides (Lewis *et al.* 2002) allowing the release of FFA, which are then able to drain directly

into the portal vein as they are from a central adipose depot rather than a peripheral adipose depot (Lewis *et al.* 2002). However in the case of the ArKO mouse no change was seen in HSL expression, even though HSL is regulated via stimulation of the catalytic activity via a cAMP-dependent mechanism (Morimoto *et al.* 2001; Okuda *et al.* 1994). Hence there may be changes posttranscriptionally. This rise in FFA can provide substrate for increased triglyceride synthesis in the liver (Bergman 1997; Haque & Sanyal 2002). Increased triglyceride synthesis likely occurred in the estrogen deficient males as seen by elevated FAS and ACCα expression, which would result in increased hepatic FA production and resulting triglyceride synthesis, thus contributing to the fatty liver phenotype. Insulin is known to inhibit HSL activity in the adipose tissue. However, when there is increased abdominal adiposity, the ability to inhibit insulin lipolysis is impaired (Chitturi *et al.* 2002; Day 2002; Youssef & McCullough 2002; Marchesini *et al.* 2001), possibly leading to further secretion of FFA from adipose tissue (Lewis *et al.* 2002), thereby leading to further production of lipids in the liver, establishing a positive feedback mechanism of disregulated FA homeostasis. In addition to insulin inhibiting HSL activity, it is also known to stimulate hepatic gluconeogenesis thereby increasing hepatic glucose output. This acts back on the pancreas, and in the case of normal pancreas function there is a further increased insulin output, resulting in normoglycaemia. This is observed in older ArKO mice, where their insulin levels are elevated, however serum glucose levels remain normal (Jones *et al.* 2000). However as described earlier, in the situation of increased abdominal obesity insulin has little effect, thereby further contributing to the lack of normal regulation of lipid homeostasis.

Based on this concept, adipose tissue serves as a buffer to the release of FFA into the circulation (Frayn 2002). When there is obesity this alters adipose tissue function and

thus makes buffering less effective as the adipocytes are filled, hence resisting further fat storage (Frayn 2002). In addition to adipose tissue the liver also has a high capacity for storage of triglycerides. Hence, if the ability of adipose tissue to store triglycerides is compromised, this then increases the need for the liver to acts as a storage centre (Gibbons *et al.* 2000). Rodents who present with lipodystrophy, where there is no adipose tissue and hence are unable to buffer FFA, add further support to this theory. In this situation there is an accumulation of triglycerides in the liver as well as skeletal muscle and pancreas (Kim *et al.* 2000; Koyama *et al.* 1997). E_2 replacement in the ArKO males, described in chapter 3, revealed a reversal of their obese phenotype by reducing gonadal, visceral and BAT fat pad weights. In addition to this there was reversal of the hepatic steatosis. This again suggests that adipose tissue may act as a buffer for triglycerides, as E_2 may have acted on the adipose depots to reduce their size and hence obviate the need for the liver to assist in the buffering of triglycerides.

Previously, we have shown that estrogen deficiency in both genders causes obesity, which is associated with an increased adipocyte volume (Jones *et al.* 2000). Estrogen replacement in the females was able to reverse this phenotype by causing a decrease in adipocyte volume, whereas there was very little change in adipocyte number (Jones *et al.* 2000; Misso *et al.* 2003b). This was also associated with a decrease in LPL expression, thereby the adipocytes are not taking up as much FFA, a possible explanation for their decrease in size.

In addition to increased synthesis of FA contributing to the hepatic steatosis, LCFA uptake may also have been increased, as seen by elevated levels of ADRP expression. This could be reversed with E_2 replacement. Additionally, ADRP has been localised

to neutral lipid storage droplets in a wide variety of cells suggesting that it plays a role in the management of neutral lipid stores (Brasaemle *et al.* 1997). Another transporter, FAT/CD36 is also affected by sex steroids (Fitzsimmons *et al.* 2002). Female rats have higher levels of protein expression compared with males, and castration of both sexes leads to comparable expression. In the study described in chapter 2, mRNA expression of FAT/CD36 was measured and there was no effect by estrogen or by gender. Hence any regulation of estrogen may possibly be posttranscriptional. These studies suggest a possible involvement of estrogen in the regulation of FFA transport.

Another contributing factor in the development of hepatic steatosis is transport of triglycerides out of the liver. Apo E is a lipoprotein present on the surface of VLDL. As described in section 1.36, mRNA expression was measured and there were no changes regardless of estrogen status. Additionally, preliminary data measuring MTTP showed no changes in expression. MTTP catalyses lipid transfer to the apoB polypeptide, and participates in the formation of triglyceride rich droplets present in the ER which are also able to fuse with apoB particles. Together this data suggests that there was also no increase in hepatic triglyceride output despite the increase in FA synthesis and uptake. Another group, which has generated an estrogen deficient ArKO mouse also, measured VLDL and found no changes in serum levels regardless of estrogen status (Toda *et al.* 2001), further supporting our finding.

5.3 Cholesterol Homeostasis

Like triglyceride homeostasis estrogen deficiency resulted in disrupted cholesterol homeostasis. In female mice there was increased serum cholesterol and HDL levels

and decreased hepatic cholesterol, whereas in males there was increased hepatic cholesterol and no changes in serum cholesterol or HDL levels, as described in chapter three. Transcripts of key enzymes in the cholesterol metabolism pathway were measured and estrogen deficiency had very little effect on these in males, however effects observed in females. *Cyp7a*, which encodes cholesterol 7α-hydroxylase, was significantly reduced in the state of estrogen deficiency, as was ACAT2, which is involved in cholesterol esterification. This was despite previous studies showing that estrogen is able to stimulate *Cyp7a* expression and cholesterol 7α-hyroxylase activity. These decreases in the expression of the genes, may be due to lower levels of hepatic cholesterol present in the estrogen deficient females rather than a direct effect on the genes themselves. Although there were changes in hepatic cholesterol concentration in the absence of estrogen in ArKO females, there were no changes in the expression of HMG CoA reductase between ArKO females and WT controls. As discussed in section 1.351 estrogen has been shown to affect HMG CoA reductase activity, rather than affecting transcription and this may possibly explain a lack of change at the levels of gene expression.

Considering the changes to serum and hepatic cholesterol levels in both male and female estrogen deficient mice, it was perhaps surprising to not find greater changes in expression of these enzymes, in particular the lack of changes in the LDLR despite elevated serum cholesterol. Additionally, estrogen has been shown to be quite a potent stimulator of LDLR expression (described in Section 1.352). However, antiestrogens such as tamoxifen and raloxifen were unable to inhibit LDLR expression, suggesting that estrogen withdrawal does not necessarily cause a decrease in LDLR expression.

In estrogen deficient male mice elevated hepatic cholesterol levels were unable to be reversed by E_2 replacement, (described in Chapter three). This is in contrast to hepatic triglyceride levels which were reduced by E_2 replacement. The E_2 replacement was given to the animals at 18 weeks of age for six weeks, thus suggesting that estrogen may play a role in neonatal programming of cholesterol controlling mechanisms. This has been shown in the expression of some cytochrome P450 genes (*Cyps*), namely that the absence of estrogen leads to a lack of expression of certain P450 isoforms. Furthermore the expression of some P450 isoforms can be restored by E_2 replacement in adulthood, however not all isoforms (described in Section 1.252) (Yamada *et al.* 2002). This indicates that estrogen is important for expression of certain liver enzymes, and in some circumstances it is required neonatally.

5.4 Sexually dimorphic phenotypes

Estrogen deficiency led to sexually dimorphic effects in both triglyceride and cholesterol homeostasis (described in Chapters two and four). The rodent liver is known to have sex specific properties due to different patterns of GH secretion, in that secretion of GH is pulsatile in males and females however, with have large intervals in between pulses in males and shorter frequency in females. These differing secretary patterns lead to differential expression of liver cytochrome P450s (Gustafsson *et al.* 1983a; Gustafsson *et al.* 1983b). It has been shown that in males, pulsatile secretion of GH leads to activation of JAK Kinases, which in turn phosphorylate Stat5b transcription factor, allowing it to translocate to the nucleus to activate target genes (Davey *et al.* 1999a; Davey *et al.* 1999b; Sueyoshi *et al.* 1999). Interestingly, Stat5b null mice have pale and enlarged livers (Davey *et al.* 1999a), which is similar to the ArKO males. Thus it is possible that growth hormone

deficiency may lead to hepatic steatosis via a mechanism involving Stat5b. Interestingly, growth hormone deficient men have also presented with hepatic steatosis (Takano *et al.* 1997) and in one male this was reversed by growth hormone replacement (Ichikawa *et al.* 2003). Estrogen deficiency in males but not females leads to cell death occurring in the arcuate nucleus (Arc) and medial preoptic area (MOP) regions of the hypothalamus (Hill *et al.* 2003). Whether or not this leads to a disruption to growth hormone secretion in the male ArKO mice is currently being investigated.

In addition to the ArKO model, other models of estrogen deficiency have shown alterations in sex-specific cytochrome P450s. In the αERKO female mice there was repression of the female-specific *Cyp2a4* gene and subsequent expression of the male specific *Cyp2d9* gene, which also remained expressed in αERKO males. Additionally, the transcription factor Stat5b had nuclear localisation in both genders of the αERKOs, where normally this would only occur in males, which is due to the differences in growth hormone secretion. When the mice were hypophysectomised Stat5b was undetectable in liver extracts of female αERKOs, indicating this regulation is through growth hormone (Sueyoshi *et al.* 1999). This study implies that ERα plays a key role in regulating growth hormone secretion in both genders and hence may a play a role regulating the sexual dimorphic expression of hepatic P450s.

Additionally, one of the ArKO mouse models revealed that the absence of estrogen led to disruptions to hepatic P450 expression (described in Section 1.252), adding further evidence to the importance of estrogen signalling for normal hepatic enzyme expression.

Obesity in both genders leads to increased leptin secretion (Jones *et al.* 2000). Leptin is a peptide hormone that is secreted from adipose tissue and signals to the leptin receptors that are present in the hypothalamus to suppress food intake. Additionally, leptin is known to regulate spontaneous physical activity (Zhang *et al.* 1994) and more recently has been shown to inhibit lipogenesis, cholesterol synthesis and to stimulate fatty acid oxidation (Muoio & Lynis 2002). Leptin resistant and deficient mouse models also present with hepatic steatosis. As mentioned earlier leptin signals to the leptin receptors present in the hypothalamus and specifically the Arc and ME regions which contain the highest concentration of leptin receptors in the brain (Sainsbury *et al.* 2002). This is one of the areas of the hypothalamus in the male ArKO mice where cell death is occurring. Hence it is possible that despite the elevated serum leptin levels in the estrogen deficient males, leptin signalling may be reduced, contributing to the hepatic steatosis in the ArKO mice.

5.5 Effects of a high cholesterol diet

In an attempt to examine the role of estrogen in regulating cholesterol homeostasis, I challenged the mice with high cholesterol diets and found some very surprising and interesting findings. Despite the disruptions to cholesterol homeostasis in the absence of estrogen, namely that males had elevated hepatic cholesterol levels and females presented with elevated serum cholesterol levels, the addition of cholesterol to the diet had much the same effect within genders, regardless of estrogen status (summarised in Table 2 Chapter four).

The high cholesterol diet had a greater effect on triglyceride homeostasis (summarised in Table 2 in Chapter two), namely it led to the reversal of the obese phenotype in both genders and of the hepatic steatosis in the ArKO males, due to a decrease in

hepatic triglycerides. Cholesterol feeding had no significant effect on genes involved in lipogenesis in either sex, therefore this was not the mechanism which lead to the reversal of the hepatic steatosis. The fatty acid transporters in males were affected differently by the high cholesterol diet and depended on estrogen status. Specifically, ADRP expression was increased by cholesterol feeding only in estrogen deficient males. Conversely, expression of CD36/FAT was reduced by cholesterol feeding only in estrogen-deficient males. The transporters FATP2 and FATP5 were only altered by cholesterol feeding in estrogen-replete males. Sexual dimorphism was also observed here, in that FATP5 expression was reduced in females after cholesterol feeding regardless of estrogen status. Hence estrogen and gender play important roles in affecting the ability of cholesterol to regulate fatty acid transport. These changes however, do not explain the mechanisms which have led to the reversal of the hepatic steatosis in the ArKO males. As proposed earlier, the hepatic steatosis is secondary to the obesity and insulin resistance. Cholesterol feeding also led to a reduction in body weight, which was reflected by a decrease in gonadal fat pad mass and therefore it is possible that cholesterol is having its effects on the adipose tissue to reverse the obese phenotype, and hence prevent the hepatic steatosis. This reduction of gonadal fat pad mass was due to a decreased adipocyte volume reflected in a decrease in LPL expression (Misso *et al.* 2003a). Smaller adipocytes may in this case therefore lead to reduced FFA to be taken up by the liver, thereby reversing the hepatic steatosis.

5.6 Conclusions

Estrogen deficiency in both males and females leads to a disruption in lipid homeostasis. Interestingly, despite estrogen deficiency in both genders, the mechanisms of these disruptions are sexually dimorphic. Furthermore, estrogen

deficiency in males leads to a more dramatic phenotype when examining triglyceride homeostasis. Therefore the work presented in this thesis has further extended the notion that estrogen is critical for lipid homeostasis, and highlighted its importance in males.

5.7 Chapter Five Bibliography

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Appendix Section

Appendix one: Cholesterol feeding prevents adipocyte hypertrophy in the obese female aromatase knockout (ArKO) mouse.
Submitted to Endocrinology, In Review

Declaration for Thesis Appendix one

In the case of Appendix one, contributions to the work involved the following:

Name	% contribution	Nature of contribution
Marie Misso	86%	All experimental work Manuscript preparation
Kylie Hewitt	3%	allocation of animals to diets, harvesting of animals HMG CoA reductase and LDLR primer design and optimisation
Wah Chin Boon	2%	Experimental assistance, Light cycler
Yoko Murata	2%	Experimental assistance, Light cycler
Margaret Jones	2%	Assistance with morphological analysis
Evan Simpson	5%	Supervisor and editing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Prince Henry's Institute of Medical Research** **PO Box 5152, MMC** **Clayton, Victoria, Australia, 3168**

Signature 1	[redacted]	**Date** 6/11/03
Signature 2		30/10/03
Signature 3		30/10/03
Signature 4		30/oct/03
Signature 5		31/10/03
Signature 6		30/10/03

Cholesterol feeding prevents adipocyte hypertrophy in the obese female aromatase knockout (ArKO) mouse.

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Abbreviated Title: Cholesterol prevents adiposity in ArKO mice.

Keywords: cholesterol, obesity, estrogen-deficiency, aromatase

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ABSTRACT

The estrogen deficient aromatase knockout (ArKO) mouse develops obesity by three months of age, marked by an increase in the weights of gonadal and infrarenal fat depots. The onset of obesity is characterised by pronounced hypertrophy of adipocytes in these mice with corresponding increases in transcripts encoding factors involved in the development of adiposity. The absence of aromatase in mice and in humans with natural mutations of the aromatase gene also leads to a metabolic syndrome in particular, hepatic steatosis. In the ArKO mouse, this hepatic steatosis along with increased body weight, surprisingly, is prevented by cholesterol feeding. In the present study we sought to investigate whether the reduction in body weight upon cholesterol feeding is reflected in gonadal fat depots since these depots account for a large percentage of body weight in the ArKO mouse. Indeed gonadal fat depots of female ArKO mice were significantly reduced upon cholesterol feeding. Concomitantly, stereological examination revealed that the adipocyte hypertrophy of the ArKO mouse was dramatically reduced upon cholesterol feeding. Transcriptional analysis using real-time PCR revealed concurrent changes with adipocyte volume in the levels of transcripts encoding lipoprotein lipase and caveolin-1. Little change was observed in levels of transcripts for factors involved in *de novo* fatty acid synthesis, β-oxidation, differentiation and cholesterol metabolism suggesting that cholesterol feeding causes a decrease in the hypertrophy of the adipocytes resulting from estrogen deficiency, primarily as a consequence of changes in levels of expression of lipoprotein lipase and therefore fatty acid uptake.

INTRODUCTION

Adipocyte hypertrophy is characterised by volume expansion of the adipocyte from excessive accumulation of intracellular lipid. The balance of three main processes determines adipocyte volume: namely, lipogenesis, lipolysis and lipid oxidation. Lipogenesis can involve *de novo* synthesis of fatty acids from acetyl CoA and employs acetyl CoA carboxylase (ACC) (1) and fatty acid synthase (FAS) (2-4). In this case the glycerol component is derived from glycerol-3-phosphate, which in turn is formed by reduction of dihydroxyacetone phosphate (4;5). Adipocyte triglycerides can also be derived from the metabolism of serum lipoproteins such as chylomicrons and very low-density lipoproteins (VLDL) (6-8), which contain apolipoprotein CII (9-11). Bound to glucosaminoglycans on the luminal surface of capillary endothelial cells (12), lipoprotein lipase (LPL) hydrolyzes the ester bonds at the 1,3 positions of the triglycerides leading to the release of free fatty acids (FFA) and *sn*2-monoglycerides, which are taken up by the adipocyte and resynthesized into new triglyceride (13). Lipolysis results in the hydrolysis of intracellular triglyceride to FFA and glycerol via the action of the enzyme hormone sensitive lipase (HSL) (13;14). These constituents can either be released into the bloodstream and taken up by the liver (15) or else the fatty acid components can be subjected to the process of mitochondrial β-oxidation which involves enzymes such as carnitine palmitoyl transferase 1 (CPT1) (16) and the long (LCAD) (17;18) and medium chain (MCAD) acyl CoA dehydrogenases (19).

There are many models of adipocyte hypertrophy, resulting from either genetic consequence, such as the Zucker (*fa/fa*) rat (20) or diet manipulation. High fat diets have been used widely, in which the development of adipocyte hypertrophy precedes the induction of obesity (21-23). Recently, a transgenic mouse model was developed, in which SREBP1a is over expressed in

adipocytes, resulting in a rise in intracellular fatty acid synthesis and secretion, thus contributing to a fatty liver (24). Morphologically, this phenotype is similar to that which we found in the aromatase knockout (ArKO) mouse. We have shown that the obese phenotype of the estrogen-deficient ArKO mouse is characterised by pronounced adipocyte hypertrophy in gonadal fat depots and further that this was attributed predominantly to LPL action in females (25). Further metabolic studies in our ArKO mouse reveal the presence of hypercholesterolemia, hypertriglyceridemia and hyperleptinemia in male and female mice (26). This obese model also displays hepatic steatosis in males, which along with increased body weight, surprisingly, is prevented upon cholesterol feeding (27).

Since gonadal fat accounts for a large percentage of body weight in the ArKO mouse (26), we sought to determine whether the reduction in body weight upon cholesterol feeding is also reflected in gonadal fat depots. Adipocyte volume has previously been associated with cholesterol availability, such that Le Lay et al (28) reported that cholesterol depletion induced hypertrophy of isolated rat adipocytes. To this end we examined gonadal fat depots morphologically and transcriptionally, using stereological methods as well as real-time PCR to investigate the mechanisms involved in cholesterol and lipid metabolism in response to cholesterol feeding in the ArKO mouse. We report here novel findings of the interaction between estrogen and cholesterol in regulating lipid metabolism in gonadal fat depots.

MATERIALS AND METHODS

Mice. ArKO mice were generated by the disruption of the aromatase gene (Cyp19) via insertion of a neomycin resistance cassette into exon 9 as described by Fisher et al (29). Heterozygous males and females were bred to produce WT and homozygous-null offspring. Mice were genotyped by PCR as described by Robertson et al (30). Animals were maintained under specific pathogen-free conditions and had unlimited access to drinking water as described (26).

Diet. At 10-12 wk of age, female WT and ArKO mice were randomly assigned to receive either control diet or high cholesterol diet for a period of 90d. The control diet consisted of a soy-free mouse chow (Glen Forest Stock Feeders, Perth, Australia) as described by Hewitt et al (31). The high cholesterol diet consisted of the soy-free mouse chow, supplemented with 2% cholesterol, which is 100 fold more than normal chow.

Tissue Collection. At 24 wk of age, mice were humanely killed by cervical dislocation. Blood was collected following decapitation and was allowed to clot. The serum was separated and stored at –20°C. Gonadal fat was removed and the wet mass measured. Of the total gonadal fat collected, 100mg was immersion-fixed in Bouins fluid, and stored in 70% alcohol at 4°C for stereological analysis; 100mg was digested for counting experiments as described below and the remainder was snap frozen in liquid nitrogen, and stored at -80°C for transcript analysis. All experiments conformed to the National Health and Medical Research Council (Australia) ethics code of practice.

Adipocyte Number. Fresh gonadal fat was digested in filtered Krebs buffer containing 8.4ml 5X salt solution (4.5% NaCl, 0.23% KCl, 0.11% KH_2PO_4, 0.19% $MgSO_4 \cdot 7H_2O$, 0.9% $CaCl_2$ in sterile H_2O), 1.3% $NaHCO_3$, 4% bovine serum albumin, 0.2% dextrose and 240U/ml collagenase in sterile H_2O. The digest was filtered through gauze to remove debris. Adipocytes were stained with methylene blue and 10µl aliquots were used for counting in a hemocytometer.

Adipocyte Volume. Bouins-fixed gonadal fat was processed in a Histokinette (Leica, Melbourne, Australia), embedded with a random orientation in paraffin and sliced into 10μm sections. Sections were stained with hematoxylin, counterstained with eosin, then cover slipped with DPX (BDH, Poole, UK). Adipocyte volume was determined at X10 magnification as described by Jones et al (26), using CASTGRID Version 1.10 (Olympus Corp., New Hyde Park, NY) on an Olympus Corp. BX50 microscope.

RNA Extraction and Quantification. Total RNA was isolated from 100mg frozen gonadal fat using the phenol/chloroform extraction method (Ultraspec RNA, Fisher Biotec, Perth, Australia). RNA was treated with ribonuclease-free deoxyribonuclease (Ambion Inc., Austin, TX). Total RNA was quantified using UV absorption at 260nm and RNA integrity was confirmed via 1% agarose gel electrophoresis.

RNA Expression. Total RNA was isolated from 100mg frozen GAT using the phenol/chloroform extraction method (Ultraspec RNA, Fisher Biotec, Perth, Australia). RNA (1µg) was reverse transcribed (RT) with Expand buffer, 10mM dithiothreitol, 20mM deoxynucleotide triphosphate mix, 20U/reaction ribonuclease I (Roche, Mannheim, Germany), 50U/reaction Expand (Roche) enzyme and sterile H_2O to a final volume of 20µl. cDNA was

diluted five times and amplified by real-time PCR in the Lightcycler (Roche) using Fast Start Master SYBR Green I (Roche) and specific oligonucleotide pairs designed to amplify a transcript that spans a minimum of two exons to avoid DNA contamination. Oligonucleotide sequences are shown in Table 1. Real-time PCR data were calculated as a ratio of transcript molecules per microgram of total RNA since we have previously demonstrated that transcripts routinely employed as internal standards are altered by the estrogen deficient state of the ArKO mouse (25).

Statistical Analysis. Study groups consisted of n=5-7. Data are expressed as mean ± SEM. Comparisons between groups were made using univariate analysis of variance and Tukey's *post hoc* test was used to determine significance (SPSS 10.0, SPSS Inc., Chicago, IL).

TABLE 1. Oligonucleotide Sequences

Transcript	Sense Primer (5'-3')	Antisense Primer (5'-3')	Product Size (bp)
LPL	AGTAGACTGGTTGTATCGGG	AGCGTCATCAGGAGAAAGG	280
FAS	CACAGATGATGACAGGAGATGG	TCGGAGTGAGGCTGGGTTGAT	205
ACCα	TGTTTGGGGTTATTTCAGTGTTGC	TGTCCAGCCAGCCAGTGTCG	236
ACCβ	CCGTGCCCTGTGCCAACCATA	GCAGCCGCTCCCCTTCATTCT	171
CPT1	ATTCTGTGCGGCCCTTATTGGAT	TTTGCCTGGGATGCGTGTAGTGT	395
LCAD	GCTGCCCTCCTCCCGATGTT	ATGTTTCTCTGCGATGTTGATG	258
SREBP2	CACAATATCATTGAAAAGCGCTACCGGTCC	TTTTTCTGATTGGCCAGCTTCAGCACCATG	200 (47)
LDL receptor	GTGGAGGAACTGGCGGCTGAAG	CTCCAGACCTCCCCATCCAGCAC	248
HMG CoA reductase	GTGGGACCAACCTTCTACCTCA	ACTGAACTGAAGCGCGGGCAT	275
Caveolin-1	CTGAGAAGCAAGTGTATGACG	CAAAGTAAATGCCCCAGATGAG	249
CD59	GACTCATCTTACTCCTGCTGCTTCT	AACACCTTTGATACACTTG	174
PPARγ Hybridisation probes	TTGACAGGAAAGACAACGGA TTTTTCAAGGGTGCCAGTTTCGATCC Flouro 3'	GAGCAGAGTCACTTGGTCATT Red 640 TAGAAGCCGTGCAAGAGATCACAGAGTATG 3'	246

RESULTS

Gonadal Fat Depots

Gonadal fat mass is significantly greater in 24-week-old ArKO mice compared with WT mice, consistent with previous findings in younger mice (26). Interestingly, cholesterol feeding resulted in a dramatic reduction in the mass of these depots to values comparable to that of WT mice (Figure 1).

Adipocyte Number

The number and volume of adipocytes determine gonadal fat mass. Adipocyte counting experiments reveal that while the number of adipocytes is unaffected by cholesterol in ArKO mice, WT mice fed a high cholesterol diet have significantly greater numbers of adipocytes than WT mice fed a control diet (Figure 2). The hyperplasia observed in gonadal fat of 10-12 wk old ArKO mice (25) is not seen in these older mice (24-week-old).

Visual Assessment of Photomicrographs

Visual examination of adipose tissue cross sections confirmed that the diameters of the adipocytes from gonadal fat of ArKO mice are much greater than those of WT, as seen in younger mice (25;26). Moreover, cholesterol feeding caused a dramatic reduction in the diameter of ArKO adipocytes from gonadal fat (Figure 3A).

Adipocyte Volume

Stereological examination of adipocyte volume confirmed that these changes seen in adipocyte diameter were characteristic of a significant increase in the volume of adipocytes from gonadal fat of ArKO mice compared to that of WT mice as shown in figure 3B. Figure 3B also

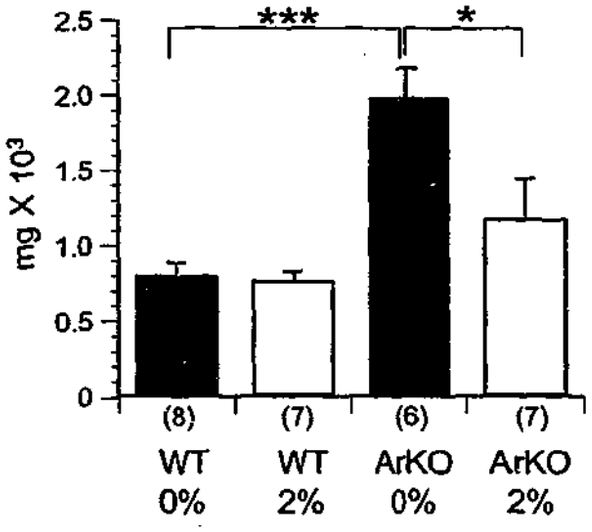

Figure 1. Gonadal Fat Mass. Gonadal adipose tissue was collected and weighed. Results ar presented as mean ± SEM. *p<0.05, ***p<0.001.

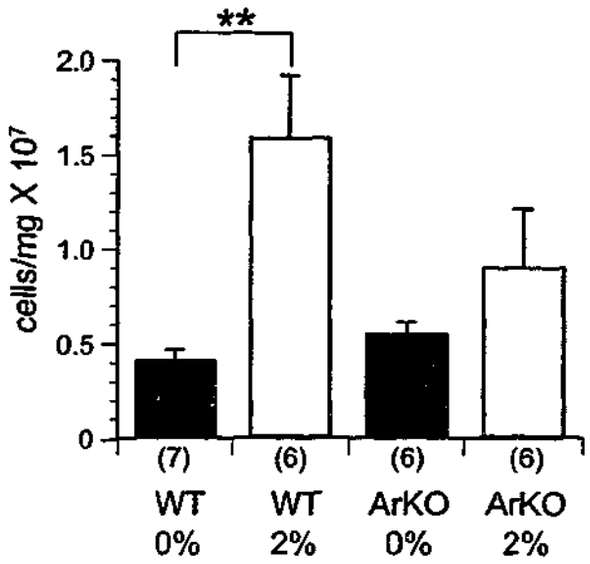

Figure 2. Adipocyte Number. Fresh gonadal adipose tissue was digested in collagenase, stained with methylene blue and adipocytes were counted with a hemocytometer. Results are presented as mean ± SEM. **p<0.01.

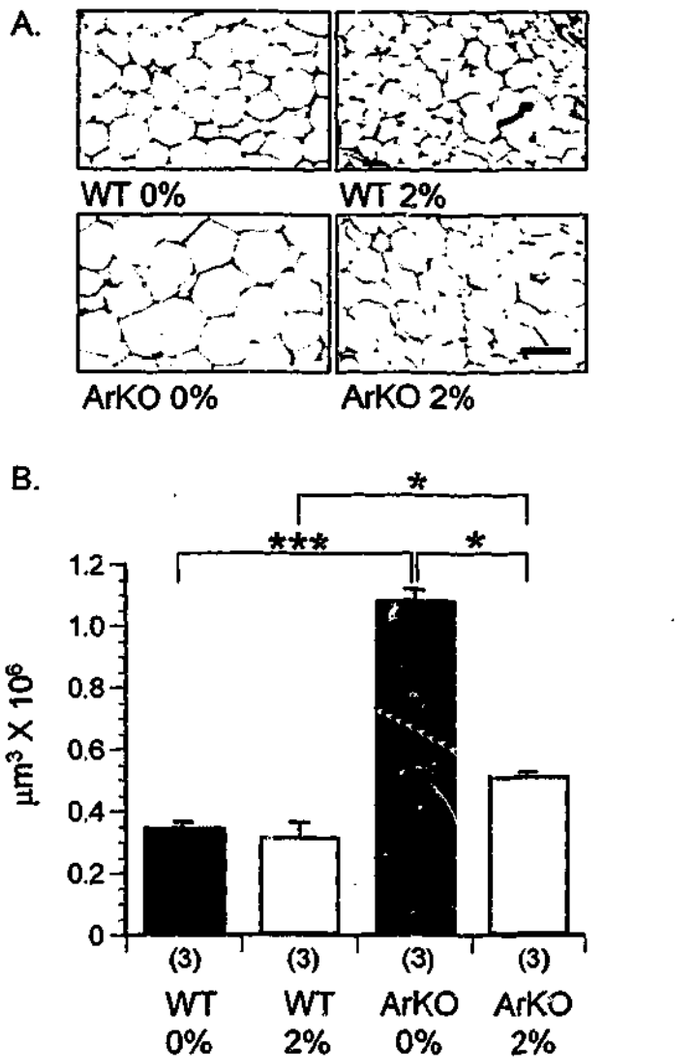

Figure 3. Adipocyte Volume. Gonadal adipose tissue was fixed in Bouins and paraffin embedded. 10µm sections were stained with Haematoxylin and Eosin. ***A.*** Adipocytes were photographed at x10 magnification. Bar represents 50µm. ***B.*** Adipocyte volume was measured using CASTGRID version 1.6 (Olympus, Denmark) in an Olympus BX50 microscope. Results are presented as mean ± SEM. *p<0.05, ***p<0.001.

demonstrates a decrease in adipocyte volume in gonadal fat of ArKO mice fed a high cholesterol diet, thus reversing hypertrophy in these mice.

Real-time PCR

In order to investigate the molecular mechanisms behind these morphological changes, real-time PCR was employed to quantify the levels of transcripts encoding enzymes and factors involved in lipid and cholesterol metabolism in gonadal fat.

Levels of transcripts encoding factors that regulate adipocyte volume

LPL is responsible for the hydrolysis of plasma triglycerides from dietary chylomicrons and very low-density proteins (VLDL) originating in the liver. This process generates FFA and *sn*2-monoglycerides that are taken up by the adipocyte. We have already shown that intracellular lipid accumulation is predominately a consequence of increased LPL expression in ArKO mice and also that estrogen treatment reduces transcript levels of LPL, resulting in loss of adipocyte hypertrophy in ArKO mice (25). In this study, we confirm that transcript levels for LPL are increased in gonadal fat depots of ArKO mice relative to WT mice. Furthermore, as shown in figure 4, levels of transcripts for LPL are reduced upon cholesterol feeding in the ArKO mice, reflecting the dramatic reduction in adipocyte hypertrophy and thus gonadal fat mass. From studies in 10-12wk old ArKO mice, we concluded that *de novo* lipid synthesis, indicated by FAS is not a factor contributing to changes in gonadal fat brought about by estrogens (25). We report here that transcript levels of FAS, ACC alpha (ACCα) and ACC beta (ACCβ) of 24-week-old ArKO mice are unchanged compared to WT controls and upon cholesterol feeding (data not shown), suggesting that de novo lipid synthesis is unchanged. Levels of transcripts encoding CPT1 and LCAD were also unchanged, similar to findings in younger mice (25) and cholesterol feeding had no effect on these transcripts, suggesting no changes in mitochondrial

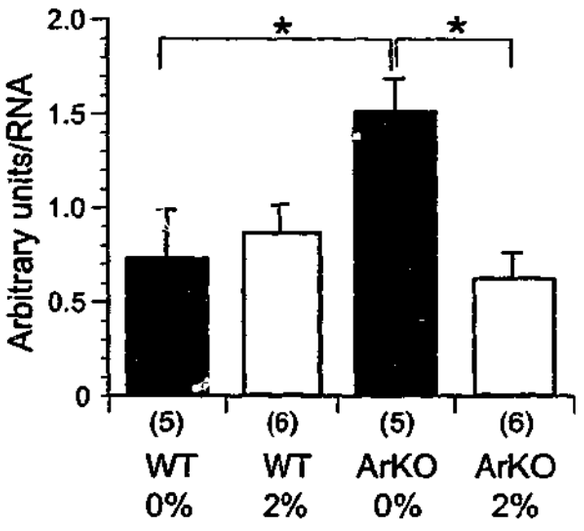

Figure 4. Lipoprotein Lipase. Total RNA was extracted from gonadal adipose tissue using the phenol/chloroform method (Ultraspec RNA, Fisher Biotec) and reverse transcribed (RT) with random hexamers. cDNA was diluted 5 times and amplified by real-time PCR in the Lightcyler (Roche), using specific oligonucleotide pairs. Results are presented as mean ± SEM. *$p<0.05$.

β-oxidation (data not shown). Again, it seems that changes in the level of transcripts of factors involved in *de novo* lipid synthesis and β-oxidation are of themselves unlikely to be responsible for the changes in hypertrophy.

Levels of transcripts for differentiation factor: peroxisome proliferator-activated receptor gamma (PPARγ)

In contrast to what we observed in younger mice (25), transcripts encoding PPARγ are unchanged in 24-week-old ArKO mice compared with WT mice and these transcripts are unaffected by cholesterol feeding (data not shown).

Levels of transcripts encoding factors involved in cholesterol metabolism

In order to determine whether cholesterol metabolism responds to cholesterol feeding in adipose tissue, we measured transcripts encoding sterol regulatory element binding protein 2 (SREBP2), 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase and the low-density lipoprotein (LDL) receptor. SREBP2 regulates intracellular cholesterol levels by stimulating transcription of the HMG CoA reductase gene resulting in increased synthesis of endogenous cholesterol and by stimulating transcription of the LDL receptor gene resulting in increased uptake of cholesterol from the circulation (31;32). We have found that in adipose tissue these transcripts are present in all four groups, however there were no significant differences observed between groups regardless of the absence of estrogen or cholesterol feeding (Figure 5), consistent with the body of data indicating that regulation of SREBP2 activity is primarily due to post-transcriptional proteolytic cleavage (33-35).

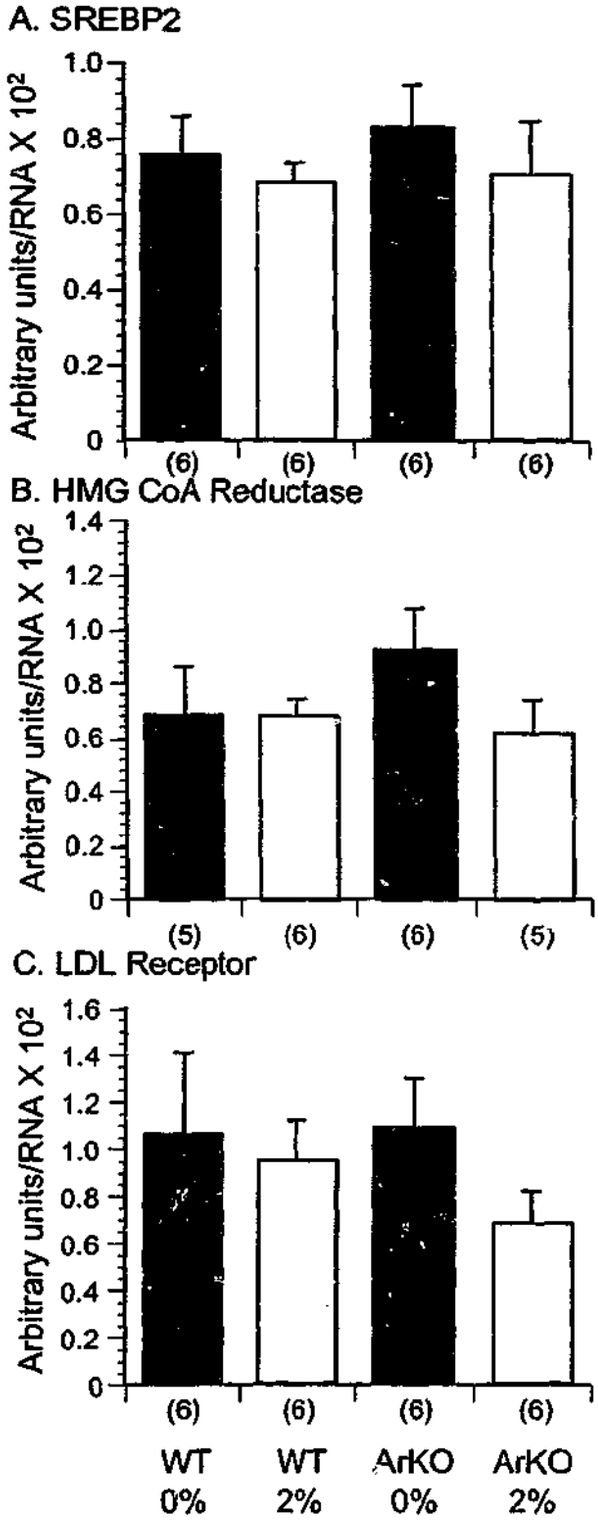

Figure 5. Factors that regulate choleterol metabolism. Total RNA was extracted from gonada adipose tissue using the phenol/chloroform method (Ultraspec RNA, Fisher Biotec) and reverse transcribed (RT) with random hexamers. cDNA was diluted 5 times and amplified by real-time PCR in the Lightcyler (Roche), using specific oligonucleotide pairs. ***A.*** Sterol regulatory elemen binding protein 2 (SREBP2) ***B.*** HMG CoA Reductase ***C.***. LDL Receptor. Results are presente as mean ± SEM.

Transcripts encoding membrane markers

Cholesterol has an important role in membrane structure and function, where it provides rigidity for caveolae and lipid rafts. Caveolin-1 is an essential component of caveolae and therefore serves as a useful membrane marker in addition to its role in intracellular trafficking (36;37). Levels of transcripts encoding caveolin-1 are significantly elevated in gonadal fat from ArKO mice compared with gonadal fat from WT mice. Upon cholesterol feeding, caveolin-1 transcripts are reduced in gonadal fat from ArKO mice (Figure 6A). These data correspond with the reported changes in adipocyte volume in these mice, where upon estrogen deficiency, the adipocytes are enlarged to a hypertrophic state together with a larger area for intracellular trafficking after which cholesterol feeding reduces their size. CD59 was also measured as a membrane marker (38), however there are no significant differences in this transcript between WT and ArKO, nor are any significant differences between the two diets (Figure 6B).

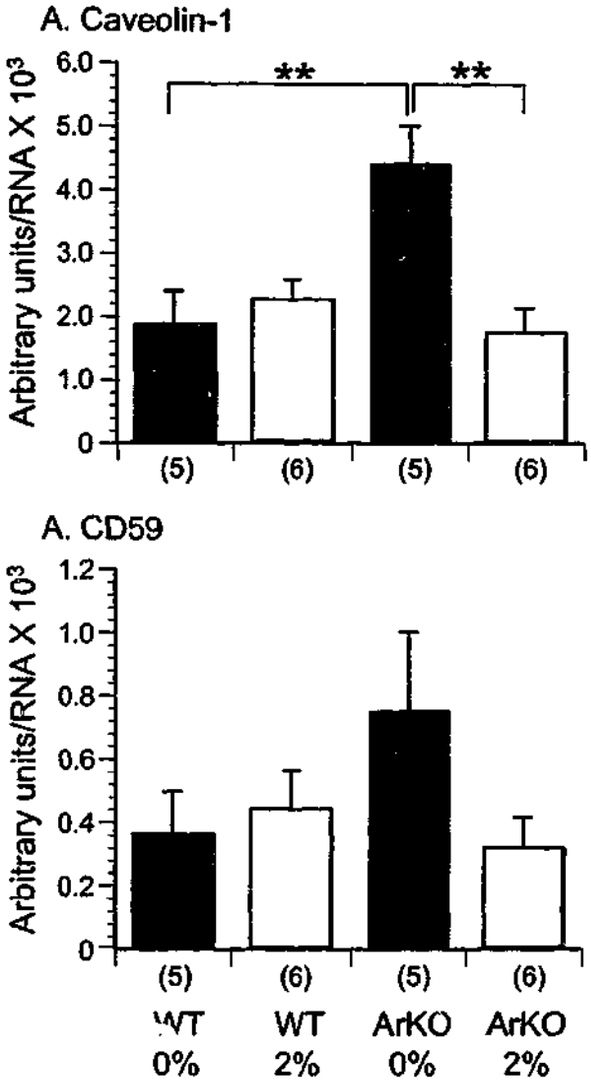

Figure 6. Membrane Markers. Total RNA was extracted from gonadal adipose tissue using the phenol/chloroform method (Ultraspec RNA, Fisher Biotec) and reverse transcribed (RT) with random hexamers. cDNA was diluted 5 times and amplified by real-time PCR in the Lightcyler (Roche), using specific oligonucleotide pairs. ***A.*** Caveolin-1 ***B.*** CD59. Results are presented as mean ± SEM. **p<0.01

DISCUSSION

Body weights of female mice (27) from all four groups are reflected in gonadal adipose tissue weights. The significant increase found in gonadal fat from ArKO mice compared with WT mice is reduced in mice fed a high cholesterol diet. We have previously reported that elevated serum cholesterol levels of ArKO mice are unchanged upon cholesterol feeding (39); hence these effects of a high cholesterol diet are not mediated by increases in circulating cholesterol levels. Concomitant with levels of transcripts encoding PPARγ, it appears that by six months of age, hyperplasia has ceased in these mice and adipocyte number does not contribute to these changes, unlike that seen in younger mice (25). Moreover, the mechanisms leading to hyperplasia observed in WT mice fed a high cholesterol diet is presently unclear but is the subject of further investigation in our laboratory. Rather, the changes in adiposity appear to be a consequence of alterations in adipocyte volume, which are in turn largely a result of changes in expression of transcripts for LPL. LPL hydrolyses plasma triglycerides from chylomicrons, releasing FFA and *sn*2-monoglycerides, which are taken up by the adipocyte, thus expanding the volume of the adipocyte, leading to hypertrophy. Interestingly, ArKO mice fed a high cholesterol diet have significantly smaller adipocytes in association with reduced transcript levels encoding LPL. These data support the early work of Lewis who reported a reduction in adipose tissue LPL activity in baboons fed a high cholesterol diet (40). We propose that LPL is the chief contributor to such changes, as enzymes involved in *de novo* fatty acid synthesis (FAS, ACCα, ACCβ), β-oxidation (CPT1, LCAD) and differentiation (PPARγ) remain unchanged between groups of WT and ArKO mice fed a control or high cholesterol diet.

We then sought to explore the status of cholesterol metabolism in the adipocyte since the adipocyte membrane requires a source of cholesterol in order to maintain structural integrity.

The integrity of cholesterol in adipocyte membranes is sustained via two mechanisms: namely, *de novo* cholesterol synthesis, from acetyl CoA and mevalonate (41), originating in the endoplasmic reticulum (42) under the direction of SREBP2 (32); and cholesterol uptake, facilitated predominantly via the LDL receptor (32;43), and may also be regulated by the VLDL receptor and LPL (44). Assuming that these processes are tightly directed as in hepatic systems, the paradigm of controlled receptor-mediated uptake and synthesis of cholesterol established by Brown and Goldstein (31;45) may apply and we might then expect that cholesterol feeding would disable SREBP2 and consequently levels of HMG CoA reductase and LDL receptor would fall. However no changes were seen in the transcript levels of these factors. Changes in the levels of transcripts for caveolin-1 are consistent with the changes in surface area that would be expected from the volume changes in the adipocytes observed in the four groups of animals, and are not elevated upon cholesterol feeding. This observation suggests that caveolin-1 levels in adipocytes are regulated primarily by changes in volume as a consequence of LPL activity and increased fatty acid uptake.

From these studies reported here, we propose that cholesterol feeding causes a decrease in the hypertrophy of the adipocytes resulting from estrogen insufficiency, and this appears to be primarily a consequence of changes in levels of expression of LPL.

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Appendix Two

Table 1: Real Time PCR Primer conditions

Primers	MgCl2 (mM)	annealing T (oC)	extension time (secs)
ABCG 5 transporter	3	62	14
ABCG 8 transporter	4	71	9
ACAT2	4	63	10
ACCα	2	60	10
ACCβ	5	60	10
ADRP	4	67	7
ApoE	3	70	9
CD36	2	68	7
Cyp7a	3	63	9
FAS	4	60	10
FATP2	4	71	5
FATP5	4	69	5
HMG CoA reductase	2	60	10
LDLR	2	60	9
MTTP	4	60	5
SR-B1	3	64	10
SREBP2	3	60	9
18s	3	60	10