0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(9):3895–3903

doi: 10.1210/en.2003-0244

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

KYLIE N. HEWITT, WAH CHIN BOON, YOKO MURATA, MARGARET E. E. JONES, AND EVAN R. SIMPSON

Prince Henry's Institute of Medical Research (K.N.H., W.C.B., Y.M., M.E.E.J., E.R.S.), Clayton, Victoria 3168, Australia; and Department of Biochemistry and Molecular Biology, Monash University (K.N.H., E.R.S.), Clayton, Victoria 3800, Australia

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 $\times$ *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).

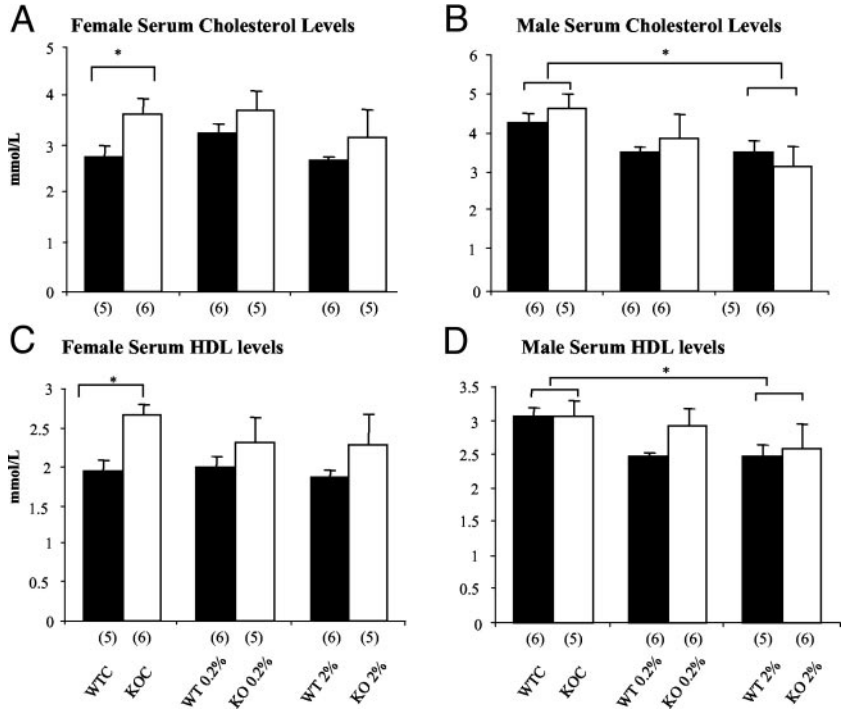

FIG. 1. Serum lipid profiles. A, Female serum cholesterol levels. Overall, ArKOs have significantly elevated serum cholesterol levels compared with WT animals ($P = 0.015$; $F_1 = 6.716$). ArKO controls have significantly elevated serum cholesterol levels compared with WT controls ($P = 0.019$; $F_1 = 6.736$). 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. *, $P < 0.05$.

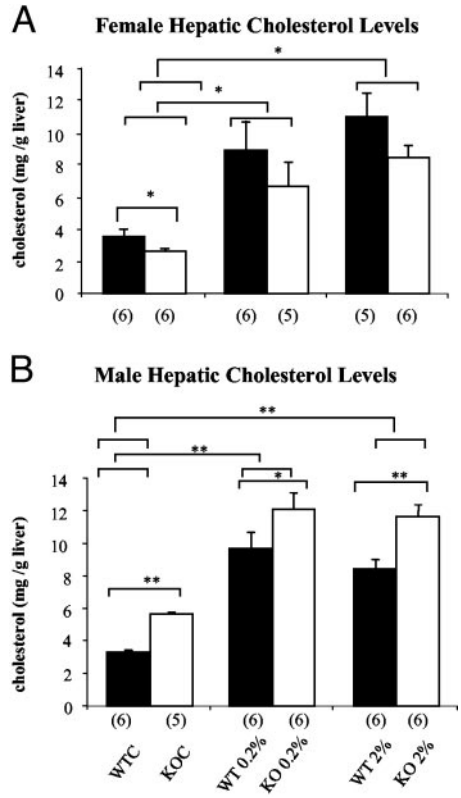

FIG. 2. Hepatic cholesterol levels. A, Female hepatic cholesterol levels. ArKO mice on the control diet have significantly lower levels of hepatic cholesterol compared with WT ($P = 0.05$; $F_1 = 4.217$). Both genotypes fed the 0.2% and 2% cholesterol diets have significantly higher levels of hepatic 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

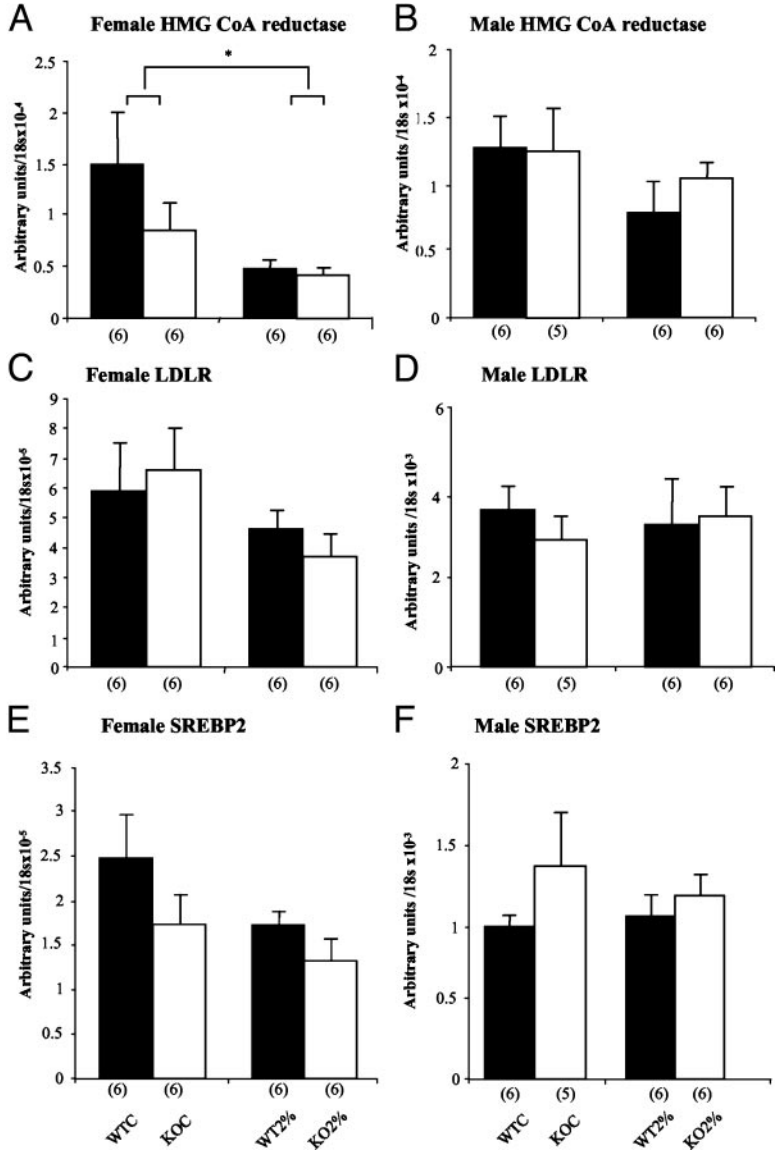

FIG. 3. Levels of transcripts for genes regulating *de novo* cholesterol synthesis and uptake. A, Female HMG CoA reductase transcript levels. Females on the control diet have significantly higher levels of transcripts for HMG CoA reductase compared with 2% cholesterol-fed females ($P = 0.017$; $F_1 = 6.766$). 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 ArKO 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

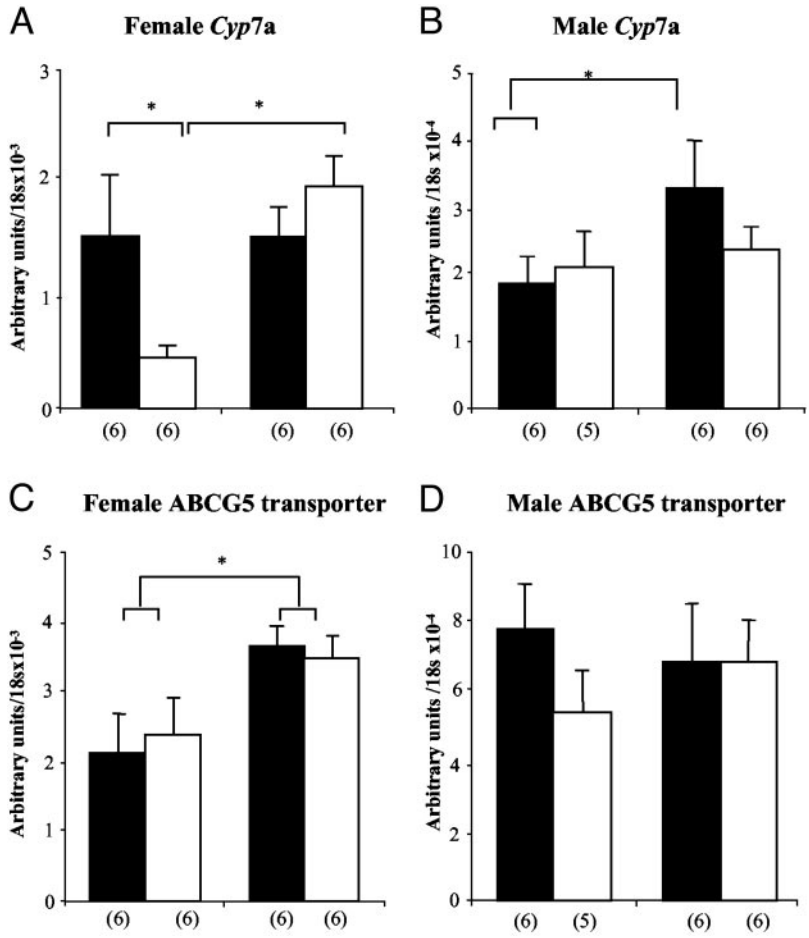

FIG. 4. Levels of transcripts for genes regulating cholesterol clearance. A, Female Cyp7a transcript levels. ArKO controls have significantly lower levels of Cyp7a compared with control WT ($P = 0.044$; $F_1 = 4.616$). ArKO females fed the 2% cholesterol diet have significantly elevated levels of Cyp7a 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

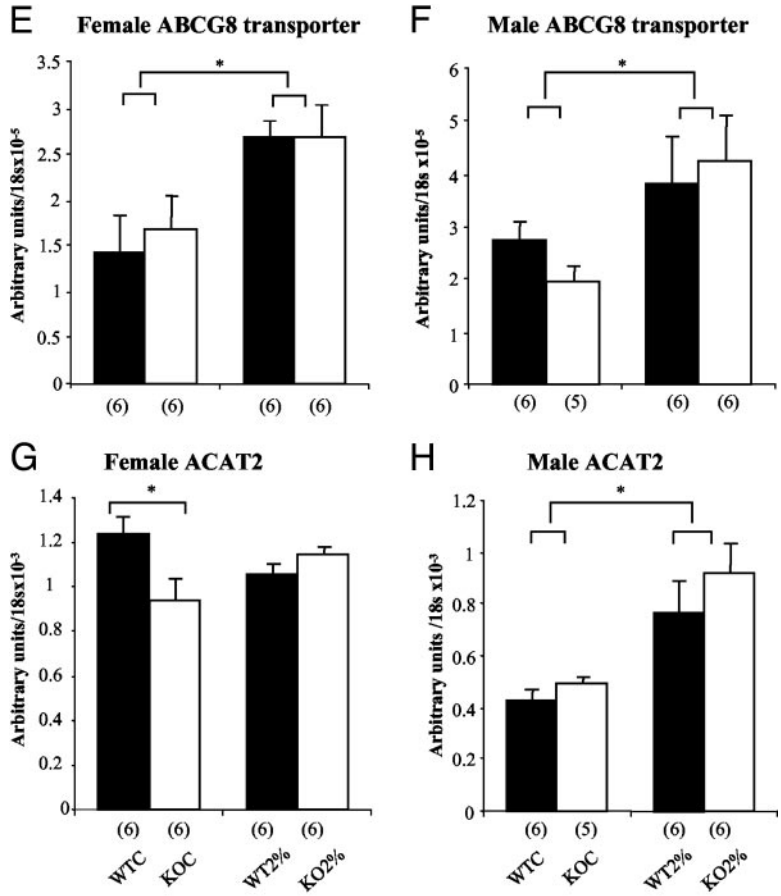

FIG. 4. *Continued*

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.

Acknowledgments

Received February 24, 2003. Accepted May 27, 2003.

Address all correspondence and requests for reprints to: Kylie Hewitt, Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton, Victoria 3168, Australia. E-mail: kylie.hewitt@med.monash.edu.au.

This work was supported by National Health and Medical Research Council Project Grant 169010 and United States Public Health Service Grant R37-AG08174.

References

1. **Heiss G, Haskell W, Mowery R, Criqui MH, Brockway M, Tyroler HA** 1980 Plasma high-density lipoprotein cholesterol and socioeconomic status. The Lipid Research Clinics Program Prevalence Study. Circulation 62:IV108–IV115
2. **Kuller LH, Gutai JP, Meilahn E, Matthews KA, Plantinga P** 1990 Relationship of endogenous sex steroid hormones to lipids and apoproteins in postmenopausal women. Arteriosclerosis 10:1058–1066
3. **Lopez D, Sanchez MD, Shea-Eaton W, McLean MP** 2002 Estrogen activates the high-density lipoprotein receptor gene via binding to estrogen response elements and interaction with sterol regulatory element binding protein-1A. Endocrinology 143:2155–2168
4. **Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB, Kagan A, Zukel WJ** 1977 HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. Circulation 55:767–772
5. **Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR** 1977 Diabetes, blood lipids, and the role of obesity in coronary heart disease risk for women. The Framingham study. Ann Intern Med 87:393–397
6. **Fisher CR, Graves KH, Parlow AF, Simpson ER** 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. Proc Natl Acad Sci USA 95:6965–6970
7. **Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O** 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166
8. **Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O** 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor β. Proc Natl Acad Sci USA 95:15677–15682
9. **Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ, Korach KS** 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. Science 286:2328–2331
10. **Jones ME, Thorburn AW, Britt KL, Hewitt KN, Wreford NG, Proietto J, Oz OK, Leury BJ, Robertson KM, Yao S, Simpson ER** 2000 Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. Proc Natl Acad Sci USA 97:12735–12740
11. **Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS** 2000 Increased adipose tissue in male and female estrogen receptor-α knockout mice. Proc Natl Acad Sci USA 97:12729–12734
12. **Ohlsson C, Hellberg N, Parini P, Vidal O, Bohlooly M, Rudling M, Lindberg MK, Warner M, Angelin B, Gustafsson JA** 2000 Obesity and disturbed lipoprotein profile in estrogen receptor-α-deficient male mice. Biochem Biophys Res Commun 278:640–645
13. **Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80:3689–3698
14. **Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER** 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337:91–95
15. **Bilezikian JP, Morishima A, Bell J, Grumbach MM** 1998 Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency. N Engl J Med 339:599–603
16. **Murata Y, Gong E, Clyne C, Aranda C, Vasquez M, Tubert G, Simpson ER,** Point mutation in the *CYP*19 gene and its consequence. Program of the 83rd Annual Meeting of The Endocrine Society, Denver, CO, 2001 (Abstract OR-11-4)
17. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams**

TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056–1061
18. **Rochira V, Balestrieri A, Madeo B, Spaggiari A, Carani C** 2002 Congenital estrogen deficiency in men: a new syndrome with different phenotypes; clinical and therapeutic implications in men. Mol Cell Endocrinol 193:19–28
19. **Brown MS, Goldstein JL** 1997 The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89:331–340
20. **Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, Mangelsdorf DJ** 1998 Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXRα. Cell 93:693–704
21. **Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ** 2002 Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors α and β. J Biol Chem 277:18793–18800
22. **Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER** 1999 Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. Proc Natl Acad Sci USA 96:7986–7991
23. **Robertson KM, O'Donnell L, Simpson ER, Jones ME** 2002 The phenotype of the aromatase knockout mouse reveals dietary phytoestrogens impact significantly on testis function. Endocrinology 143:2913–2921
24. **Gustafsson JA, Eden S, Eneroth P, Hokfelt T, Isaksson O, Jansson JO, Mode A, Norstedt G** 1983 Regulation of sexually dimorphic hepatic steroid metabolism by the somatostatin-growth hormone axis. J Steroid Biochem 19:691–698
25. **Gustafsson JA, Mode A, Norstedt G, Eneroth P, Hokfelt T** 1983 Growth hormone: a regulator of the sexually differentiated steroid metabolism in rat liver. Prog Clin Biol Res 135:37–59
26. **Hewitt KN, Boon WC, Murata Y, Simpson ER, and Jones ME,** Oestrogen deficiency in the aromatase knockout (ArKO) mouse leads to lipid accumulation in the liver. Proc Endocrine Society of Australia, Gold Coast, Australia, 2001 (Abstract 414)
27. **Spector AA** 1984 Plasma lipid transport. Clin Physiol Biochem 2:123–134
28. **Spady DK, Woollett LA, Dietschy JM** 1993 Regulation of plasma LDL-cholesterol levels by dietary cholesterol and fatty acids. Annu Rev Nutr 13: 355–381
29. **Peet DJ, Janowski BA, Mangelsdorf DJ** 1998 The LXRs: a new class of oxysterol receptors. Curr Opin Genet Dev 8:571–575
30. **Parini P, Angelin B, Rudling M** 1997 Importance of estrogen receptors in hepatic LDL receptor regulation. Arterioscler Thromb Vasc Biol 17:1800–1805
31. **Di Croce L, Bruscalupi G, Trentalance A** 1996 Independent behavior of rat liver LDL receptor and HMGCoA reductase under estrogen treatment. Biochem Biophys Res Commun 224:345–350
32. **Parini P, Angelin B, Stavreus-Evers A, Freyschuss B, Eriksson H, Rudling M** 2000 Biphasic effects of the natural estrogen 17β-estradiol on hepatic cholesterol metabolism in intact female rats. Arterioscler Thromb Vasc Biol 20:1817–1823
33. **Marino M, Distefano E, Pallottini V, Caporali S, Bruscalupi G, Trentalance A** 2001 Activation of IP_3-protein kinase C-α signal transduction pathway precedes the changes of plasma cholesterol, hepatic lipid metabolism and induction of low-density lipoprotein receptor expression in 17-β-oestradiol-treated rats. Exp Physiol 86:39–45
34. **Di Croce L, Vicent GP, Pecci A, Bruscalupi G, Trentalance A, Beato M** 1999 The promoter of the rat 3-hydroxy-3-methylglutaryl coenzyme A reductase gene contains a tissue-specific estrogen-responsive region. Mol Endocrinol 13:1225–1236
35. **Chico Y, Fresnedo O, Botham K, Lacort M, Ochoa B** 1996 Regulation of bile acid synthesis by estradiol and progesterone in primary cultures of rat hepatocytes. Exp Clin Endocrinol Diabetes 104:137–144
36. **Chico Y, Fresnedo O, Lacort M, Ochoa B** 1994 Effect of estradiol and progesterone on cholesterol 7α-hydroxylase activity in rats subjected to different feeding conditions. Steroids 59:528–535
37. **Kushwaha RS, Lewis DS, Carey KD, McGill Jr HC** 1991 Effects of estrogen and progesterone on plasma lipoproteins and experimental atherosclerosis in the baboon (*Papio sp.*). Arterioscler Thromb 11:23–31
38. **Deliconstantinos G, Ramantanis G** 1982 Evoked effects of oestradiol on hepatic cholesterol 7α-hydroxylase and drug oxidase in castrated rats. Int J Biochem 14:811–815
39. **Chao YS, Windler EE, Chen GC, Havel RJ** 1979 Hepatic catabolism of rat and human lipoproteins in rats treated with 17α-ethinyl estradiol. J Biol Chem 254:11360–11366
40. **Schneider WJ, Kovanen PT, Brown MS, Goldstein JL, Utermann G, Weber W, Havel RJ, Kotite L, Kane JP, Innerarity TL, Mahley RW** 1981 Familial dysbetalipoproteinemia. Abnormal binding of mutant apoprotein E to low density lipoprotein receptors of human fibroblasts and membranes from liver and adrenal of rats, rabbits, and cows. J Clin Invest 68:1075–1085
41. **Ma PT, Yamamoto T, Goldstein JL, Brown MS** 1986 Increased mRNA for low density lipoprotein receptor in livers of rabbits treated with 17α-ethinyl estradiol. Proc Natl Acad Sci USA 83:792–796
42. **Ueyama T, Shirasawa N, Numazawa M, Yamada K, Shelangouski M, Ito T, Tsuruo Y** 2002 Gastric parietal cells: potent endocrine role in secreting estrogen as a possible regulator of gastro-hepatic axis. Endocrinology 143:3162–3170
43. **Tannenbaum GS, Martin JB** 1976 Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. Endocrinology 98: 562–570
44. **Jansson JO, Eden S, Isaksson O** 1985 Sexual dimorphism in the control of growth hormone secretion. Endocr Rev 6:128–150
45. **Giustina A, Veldhuis JD** 1998 Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. Endocr Rev 19:717–797
46. **Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS** 1997 Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. J Clin Invest 99:838–845