Increased Exposure to Estrogens Disturbs Maturation, Steroidogenesis, and Cholesterol Homeostasis via Estrogen Receptor α in Adult Mouse Leydig Cells

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Deteriorated male reproductive health has been connected to overexposure to estrogens or to imbalanced androgen-estrogen ratio. Transgenic male mice expressing human aromatase ($AROM^+$ mice) serve as an apt model for the study of the consequences of an altered androgen-estrogen ratio. Our previous studies with $AROM^+$ mice showed that low androgen levels together with high estrogen levels result in cryptorchidism and infertility. In the present study, the $AROM^+$ mice were shown to have severe abnormalities in the structure and function of Leydig cells before the appearance of spermatogenic failure. Decreased expression of adult-type Leydig cell markers (*Ptgds, Vcam1*, *Insl3*, *Klk21*, *-24* and *-27*, *Star*, *Cyp17a1*, and *Hsd17b3*) indicated an immature developmental stage of the Leydig cells, which appears to be the first estrogen-dependent alteration. Genes involved in steroidogenesis (*Star*, *Cyp17a1*, and *Hsd17b3*) were suppressed despite normal LH levels. The low expression level of kallikreins 21, 24, and 27 potentially further inhibited Leydig cell function via remodeling extracellular matrix composition. In connection with disrupted steroidogenesis, Leydig cells showed enlarged mitochondria, a reduced amount of smooth endoplasmic reticulum, and an accumulation of cholesterol and precursors for cholesterol synthesis. The results of studies with $AROM^+$ mice crossed with estrogen receptor α or β (ERα and ERβ, respectively) knockout mice lead to the conclusion that the structural and functional disorders caused by estrogen exposure were mediated via ERα, whereas ERβ was not involved. **(*Endocrinology* 150: 2865–2872, 2009)**

Estrogen excess, especially during the fetal or neonatal period, has been connected to several male reproductive disorders, such as cryptorchidism, epididymal defects, impaired fertility, and increased incidence of testicular cancer (1). However, the androgen-estrogen balance has been postulated to be more important than absolute levels of estrogens or androgens for male fertility (2) and for reproductive tract function (3, 4).

Estrogens have been suggested to control Leydig cell function at various stages of development. Prenatal estrogen treatment has been shown to affect the differentiation of fetal Leydig cells (5), and the regeneration of Leydig cells was inhibited by estrogens in a rat model mimicking postnatal Leydig cell development (6). Several studies also indicated a role for estradiol (E2) as a paracrine/autocrine factor in the regulation of steroidogenesis in the Leydig cells, and a synthetic estrogen, diethylstilbestrol, has been shown to inhibit 17α-hydroxylase activity in the mature mouse testis (7). This was later confirmed in studies showing that estrogens regulate 17α-hydroxylase gene expression in the postpubertal (8) and fetal (9) rodent testis. Recent studies with mice lacking functional estrogen receptor (ER)α or -β [ER knockout (ERKO) and BERKO, respectively] suggest that regulation of steroidogenic genes in fetal Leydig cells is, at least partially, me-

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Abbreviations: BERKO, ERβ Knockout; E2, Estradiol; ECM, extracellular matrix; EM, electron microscopy; ER, estrogen receptor; ERKO, ERα knockout; IGFBP-3, IGF-binding protein-3; qRT-PCR, quantitative real-time RT-PCR; SER, smooth endoplasmic reticulum; T, testosterone; WT, wild type.

diated via ERα (10, 11). Estrogen has also been shown to inhibit 17α-hydroxylase and hydroxysteroid (17β) dehydrogenase type 3 gene expression through ERα in the mouse ovary (12, 13).

Normal steroidogenesis in Leydig cells requires the controlled expression and function of steroidogenic genes together with proper transport of steroid precursors between the inner and outer membrane of mitochondria and from mitochondria to the smooth endoplasmic reticulum (SER). It is well known that the first and rate-limiting step in steroidogenesis, *i.e.* transport of cholesterol from the outer to the inner membrane of mitochondria is initiated by steroidogenic acute regulatory protein (StAR) (14). Recently, it has been shown that in addition to providing matrix for the first step in steroidogenesis, mitochondria have an important regulatory role in steroidogenesis. Allen and co-workers (15) showed that to support LH-dependent steroidogenesis, mitochondria must be properly energized and polarized and be actively respiring. Furthermore, studies by Midzak and co-workers (16) indicated that inhibition of the mitochondrial electron transport chain blocks LH-dependent but stimulates basal testosterone (T) production in mouse Leydig cells. Although the effects of estrogens on mitochondrial function have been studied in several ER target cells, *e.g.* in human breast cancer cells and brain endothelial cells (17, 18), there are no previous studies reporting the effects of estrogens on Leydig cell mitochondria.

We have previously shown that male mice universally expressing low levels of human P450 aromatase gene ($AROM^+$ mice), thus having a low circulating and intratesticular androgen-estrogen ratio, are infertile, cryptorchid, and have Leydig cell hypertrophy and hyperplasia (19). In addition, chronic inflammation characterized by a high amount of activated macrophages, mast cell infiltration, and interstitial fibrosis are evident in the testis of aged $AROM^+$ mice (20). Interestingly, similar inflammation-related changes are observed in the testes of infertile men (21–23). In the present study, we demonstrate that chronic imbalance in the androgen-estrogen ratio leads to severe abnormalities in the development, structure, and function of mouse Leydig cells. The deleterious effects of estrogens were, furthermore, shown to be ERα mediated, whereas ERβ has no role in the process.

Materials and Methods

Animals and experimental groups

Generation of transgenic mice expressing human aromatase cDNA under the control of ubiquitin C promoter ($AROM^+$ mice) has been previously described (19). ERKO mice in C57BL/6J background (24) and BERKO mice in C57BL/6J/129 hybrid background (25) were crossbred to FVB/N background for seven generations. Thereafter, $AROM^+$ mice (in FVB/N background) were crossbred either with ERKO or BERKO mice. Genotyping of the mice was carried out as previously described (19, 24, 25). The mice had free access to soy-free food pellets (SDS; Witham, Essex, UK) and tap water. All animal experimentation was conducted in accordance with the institutional animal care policies of the University of Turku (Turku, Finland). Animal experiments were approved by the respective authorities, and the institutional policies on animal experimentation fully met the requirements as defined in the National Institutes of Health guide on animal experimentation.

Expression array

A genome-wide gene expression study from the testis was performed using Sentrix Mouse-6 Expression BeadChip microarrays (Illumina Inc., San Diego, CA). The testes of three 3-month-old $AROM^+$ mice were compared with three age-matched wild-type (WT) controls. Total RNA was isolated from testes using Trizol reagent (Invitrogen, Carlsbad, CA) as homogenization solution and purified further with the RNeasy RNA isolation mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Two micrograms of total RNA were converted to cDNA and subsequently to cRNA (including biotinylated UTP) using the Illumina TotalPrep RNA Amplification Kit. The cRNA was further hybridized to the Sentrix Mouse-6 Expression BeadChip microarray chip as instructed by the manufacturer. After hybridization, the fluorescent signal was quantified and analyzed using an Illumina Bead Array Reader. The quality of the experiment was controlled by the Illumina Bead Scan program (Illumina Inc., San Diego, CA). The data from the Illumina arrays were analyzed with the Knowledge Discovery Environment (KDE) program (InforSense, London, UK) and statistical programming language R. The data were first normalized with the Quantile method to remove technical variation. After normalization, the data were analyzed by calculating the mean values, logarithmic intensity ratio

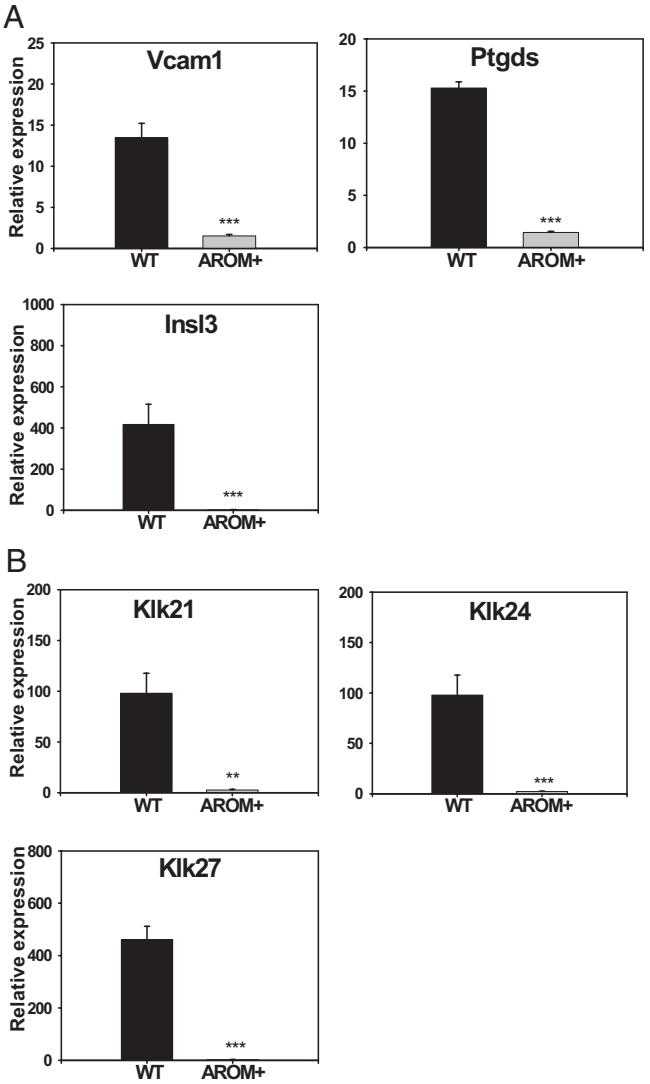

FIG. 1. Down-regulation of the expression of adult-type Leydig cell markers in 3-month-old $AROM^+$ mouse testis. The expression of *Vcam1*, *Ptgds*, and *Insl3* (A) and *Klk21*, *Klk24*, and *Klk27* (B) was significantly down-regulated in $AROM^+$ mouse testis as compared with the age-matched WT testis. n = 6 in all study groups. **, $P \leq 0.01$; ***, $P \leq 0.001$.

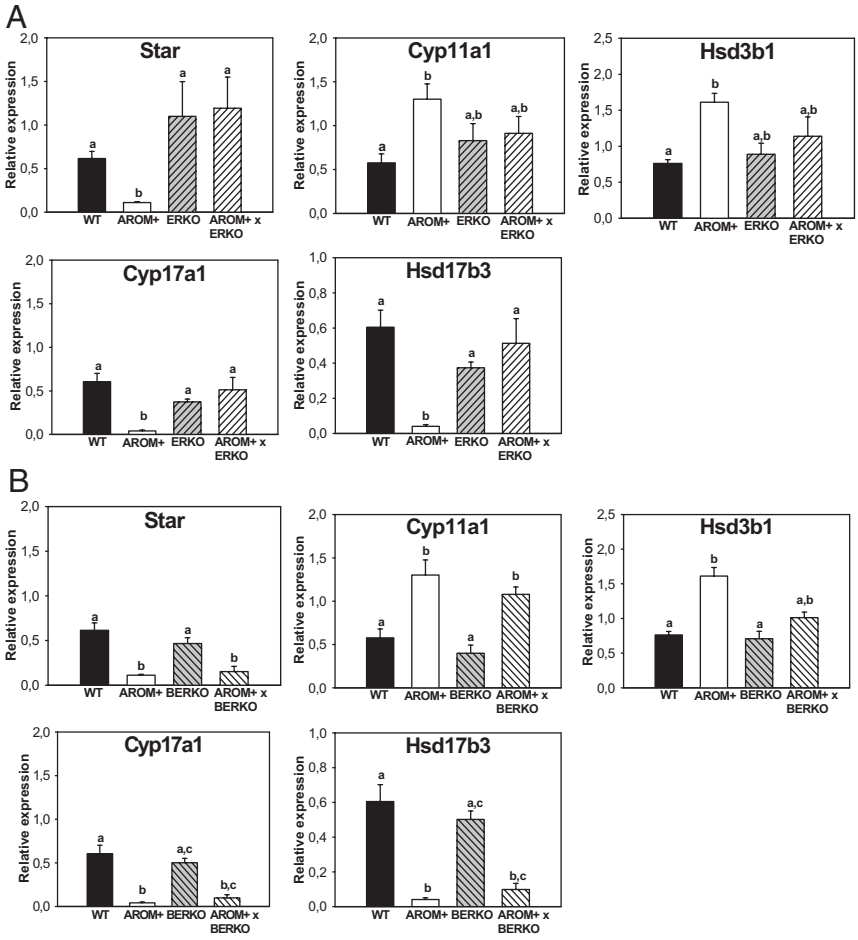

FIG. 2. A, The down-regulation of steroidogenic gene expression, *Star*, *Cyp17a1*, and *Hsd17b3*, in the AROM$^+$ mouse testis was ERα dependent, because the expression levels were normalized to the WT levels in the AROM$^+$ mice crossbred with the ERKO mice. B, The lack of ERβ in the AROM$^+$ mice had no effect on the expression of *Star*, *Cyp17a1*, and *Hsd17b3* in the AROM$^+$ mice. The expression levels of *Cyp11a1* and *Hsd3b1* were slightly higher in the AROM$^+$ mice probably due to the increased relative number of Leydig cells in the AROM$^+$ mice as compared with the WT mice (A and B). There is a statistically significant difference ($P \leq 0.05$) between the groups provided with *different letters*. n = 6 in all groups studied.

(logR), logarithmic mean intensity (A), and fold change (FC) for two experimental groups. The statistical significances of the differences in gene expression values (Log2 transformed) between AROM$^+$ and WT testes were analyzed using the *t* test. Only the genes that were over 2.0-fold up- or down-regulated in the AROM$^+$ group and having a *P* value < 0.05 were listed.

Quantitative real-time RT-PCR (qRT-PCR)

The expression of adult-type Leydig cell marker and steroidogenic enzyme genes was analyzed by qRT-PCR. Total RNA was isolated from testes using the RNeasy mini kit (QIAGEN). One microgram of total RNA was thereafter treated with deoxyribonuclease (DNase I Amplification Grade Kit; Invitrogen Life Technologies, Paisley, UK). cDNA synthesis and RT-PCR were performed using the DyNAmo two-step SYBR Green qRT-PCR kit (Finnzymes Oy, Espoo, Finland). The expression levels were analyzed in proportion to the L19, β-actin, and cyclophilin housekeeping genes. The primer sequences used in this study are available on request.

Hormone measurements

Serum LH and FSH levels were measured by using time-resolved immunofluorometric assays as described previously (26). For intratesticular T and E2 measurements, the testes were weighed and homogenized in Dulbecco's PBS. E2 and T were extracted from the homogenates using diethyl ether. After extraction, the organic phase was evaporated to dryness, and the steroids were solubilized in PBS with 0.1% BSA. E2 concentrations were measured by an immunofluorometric assay, using the human E2 DELFIA kit (PerkinElmer Wallac Oy, Turku, Finland) adapted for mouse samples. T levels were measured by a RIA as described previously (27).

Electron microscopy studies

Whole testes were fixed for 2 h in 5% electron microscopy (EM)-grade glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in PBS after which they were cut into four pieces. The samples were then fixed for an additional 2–3 d at +4 C and then stored in PBS at +4 C. As described previously (28, 29), samples were postfixed with 4% OsO_4/potassium hexacyanoferrate (II). After embedding in Epon, semi-thin and ultra-thin sections were cut, contrasted with uranyl acetate (2%)/lead citrate (2.7%), and examined with an EM10 electron microscope (Zeiss, Jena, Germany).

Lipid measurements

The testes were weighed, cut with scissors into small pieces, and homogenized in chloroform/methanol with Ultra Turrax after the addition of internal standards 5α-cholestane and epicoprostanol. After homogenization, sterols were first subjected to analysis by thin-layer chromatography. Esterified sterols, with 5α-cholestane, were extracted from the front area of the thin-layer chromatography plate, and free sterols were extracted from the lower area of the plate, including free cholesterol, plant sterols, methyl sterols, and epicoprostanol. The esterified sterol fraction was saponified, subjected separately with the free sterol fraction to gas liquid chromatographic (GLC) analysis as trimethylsilyl-derivatives, principally as described earlier (30) using a 50-m-long Ultra 2 capillary column (Agilent Technologies, Wilmington, DE). Concentrations of cholesterol, cholestenol, lathosterol, desmosterol, and lanosterol were measured by the gas liquid chromatographic procedure in increasing order of retention time. The coefficients of variation for the different sterols were as follows: cholesterol 3.2%, desmosterol 6.0%, and lathosterol 3.7%.

Statistical analysis

Statistical analyses (from other than microarray results) were performed by the SigmaStat program (version 3.1 for Windows 2000 and XP; SPSS Inc., Chicago, IL). Student's *t* test or the Mann-Whitney rank sum test was performed for analyzing the statistical significance ($P < 0.05$) between two groups, whereas one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks was applied for analyzing several groups.

Results

A genome-wide expression array was performed at the age of 3 months. At this age, the spermatogenesis in the AROM$^+$ mice was qualitatively normal based on testicular morphology, whereas it was severely disrupted at a later age. The data indicated that 30 genes were down-regulated in the testis of AROM$^+$ mice as compared with WT mice (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals

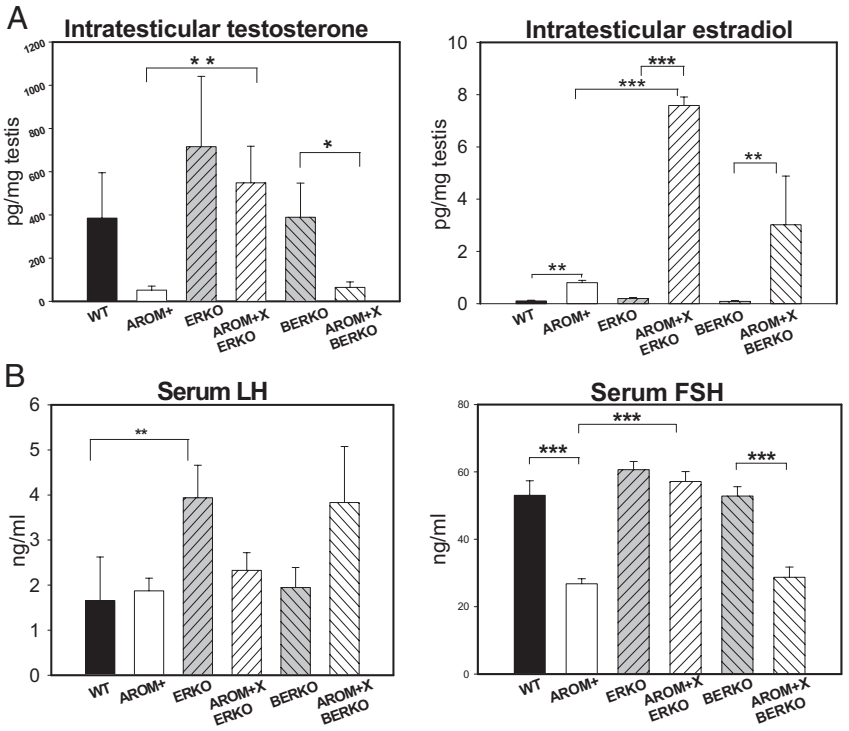

FIG. 3. Serum (A) and intratesticular (B) hormone concentrations in different mouse groups. Serum LH levels were significantly elevated in the ERKO mice as compared with the WT mice. Serum FSH concentration was significantly lower in the AROM$^+$ mice as compared with the WT group, and crossing with the ERKO mice corrected the concentration to the WT level. Intratesticular T levels were lower (not significantly) and E2 higher in the AROM$^+$ mice as compared with the WT mice. Crossing of the AROM$^+$ mice with the ERKO mice further increased E2 levels, whereas T levels were rescued to the WT levels. Lack of ERβ had no effect on hormone levels. Pairwise Student's *t* test or the Mann-Whitney rank sum test was performed to analyze statistical significance as follows: WT *vs.* AROM$^+$, WT *vs.* ERKO, WT *vs.* BERKO, AROM$^+$ *vs.* AROM$^+\times$ ERKO, AROM$^+$ *vs.* AROM$^+\times$ BERKO, ERKO *vs.* AROM$^+\times$ ERKO, and BERKO *vs.* AROM$^+\times$ BERKO. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. n = 6 in all study groups.

Online web site at http://endo.endojournals.org). The AROM$^+$ mice presented with low-level expression of several marker genes for maturated adult-type Leydig cells including *Insl3* (13.3-fold), *Ptgds* (7.4-fold), and *Vcam1* (2.8-fold). In addition, the expression of steroidogenic enzymes *Hsd17b3* (2.5-fold) and *Cyp17a1* (2.1-fold) and Leydig cell-specific extracellular matrix (ECM) proteinases *Klk21*, *Klk24*, and *Klk27* (8.5-, 6.5-, and 4.6-fold, respectively) were significantly lower in AROM$^+$ mice. The genes marked with *asterisks* in supplemental Table 1 were recently shown to be down-regulated by estrogen also in the fetal-type Leydig cells (11). Low-level expression of *Insl3*, *Vcam1*, *Ptgds*, *Klk21*, *Klk24*, and *Klk27* expression was further confirmed by qRT-PCR analysis (Fig. 1). These data indicate an immature developmental stage of Leydig cells in young adult AROM$^+$ males.

qRT-PCR analyses showed that the expression of *Star*, *Cyp17a1*, and *Hsd17b3* was significantly decreased in young AROM$^+$ testes as compared with the WT mice, and the expression was normalized to the WT level in AROM$^+$ mice crossbred with ERKOs (Fig. 2A). In contrast, the gene expression was not normalized by crossing the AROM$^+$ mice with the BERKO mice (Fig. 2B), suggesting that down-regulation of the steroidogenesis in AROM$^+$ testis is estrogen dependent and particularly ERα mediated. The elevated expression levels of *Cyp11a1* and *Hsd3b1* in the AROM$^+$ mice (Fig. 2, A and B) probably reflect the increased relative number of Leydig cells in AROM$^+$ mice as compared with the WT mice. The down-regulation of the expression of *Star*, *Cyp17a1*, and *Hsd17b3* in AROM$^+$ Leydig was not explained by decreased serum LH levels. Only the ERKO mice had significantly increased LH levels, as has been previously shown (31, 32). Because of the enforced expression of the transgenic P450arom, the concentration of intratesticular E2 was significantly higher in the AROM$^+$ as compared with the WT mice, and crossing the AROM$^+$ mice with the ERKO mice further elevated the E2 levels, in line with the normalized T and FSH production in the AROM$^+$/ERKO mice. Lack of ERβ had no effect on the hormone levels either in WT or in AROM$^+$ mice (Fig. 3).

Based on histological data, spermatogenesis was normal in all groups studied. However, in the ERKO mice, the tubules were slightly dilated (Fig. 4, C and D) as has been previously reported (33). In the AROM$^+$ mice, there were obvious changes in the interstitial tissue, *i.e.* hypertrophic Leydig cells (Fig. 4B). Similarly, hypertrophic Leydig cells were present in the interstitial tissue of the AROM$^+$ mice crossbred with the BERKO mice (Fig. 4F) but not in the AROM$^+$ mice crossbred with the ERKO mice (Fig. 4D). Thus, the Leydig cell hypertrophy in AROM$^+$ testis is ERα mediated. EM of AROM$^+$ testis further indicated ultrastructural changes in Leydig cells, *i.e.* the mitochondria were strikingly enlarged, SER was barely visible, and lipid accumulation (with the presence of cholesterol crystals) was evident (Fig. 5A). These results were also apparent in the AROM$^+$ mice crossbred with the BERKO mice but not in the AROM$^+$ mice crossbred with the ERKO mice. Together with light microscopy, the EM results show that the ultrastructural changes in the AROM$^+$ Leydig cells were ERα mediated (Fig. 5, D and E).

Lipid measurements from testis homogenates indicated that concentration of cholesterol, especially cholesterol ester, was significantly increased in the testes of the AROM$^+$ mice as compared with those of the WT mice (Table 1). In contrast, the serum cholesterol levels were decreased in the AROM$^+$ mice (data not shown). Furthermore, several intermediates of cholesterol biosynthesis (cholestenol, desmosterol, lathosterol, and lanosterol) (34, 35) were markedly accumulated in the AROM$^+$ mouse testis (Table 1). The data thus suggest that *de novo* cholesterol biosynthesis is increased in the Leydig cells of AROM$^+$ mice.

Discussion

We have previously shown that a low androgen-estrogen ratio in male mice expressing human aromatase gene under ubiquitin C

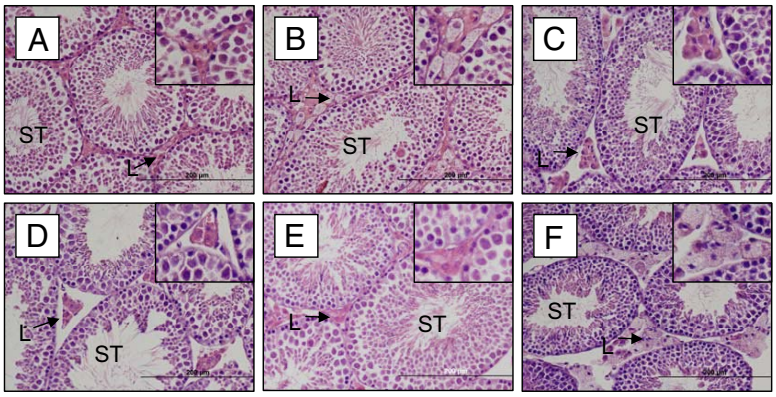

FIG. 4. Histological staining of the testes of 3-month-old mice demonstrated that the Leydig cell hypertrophy in the AROM$^+$ mice is ERα dependent. At the age of 3 months, the hypertrophy of the Leydig cells (*arrow*) is the only apparent histological phenotype in AROM$^+$ (B) as compared with the WT (A). The Leydig cells of ERKO (C), AROM$^+$ crossed with ERKO (D), and BERKO (E) mice had no signs of hypertrophy, whereas the Leydig cells of AROM$^+$ crossed with BERKO (F) mice resembled those of the AROM$^+$ mice. Leydig cells are presented with a higher magnification in the *upper right corners*. L, Leydig cell; ST, seminiferous tubule. *Bar*, 200 μm.

promoter (AROM$^+$ mice) results in infertility and cryptorchidism (19). This testicular dysfunction develops progressively; spermatogenesis is normal immediately after puberty but is destroyed during ageing in association with macrophage and mast cell infiltration and activation (20). However, as shown in the present study, the first estrogen-induced molecular and structural changes in the testis of young adult AROM$^+$ mice appear in the Leydig cells that are hypertrophic and present with disrupted maturation and reduced steroidogenic capacity associated with markedly enlarged mitochondria.

The expression of the *Insl3*, *Ptgds*, *Vcam1*, *Star*, *Cyp17a1*, and *Hsd17b3* genes is often used as a marker for adult, maturated Leydig cells (36–38). The expression of the above mentioned markers was lower in the AROM$^+$ testis at the age of 3 months, suggesting that the maturation of Leydig cells is not completed. Lipid accumulation that was evident in the Leydig cells of the AROM$^+$ mice is also a characteristic feature of immature adult Leydig cells (39). Our studies thus support the hypothesis that estrogens act as paracrine/autocrine factors in controlling adult-type Leydig cell development (40).

In addition to the elevated E2 levels caused by overexpression of P450arom, the AROM$^+$ mice have low intratesticular and circulating T levels, although LH concentrations are normal (19, 41). In the present study, only ERKO mice had increased LH levels, which is in line with the previously observed reduction in feedback regulation due to the lack of ERα in the central nervous system (31, 32). Interestingly, in AROM$^+$, AROM$^+$/ERKO, and AROM$^+$/BERKO mice, the LH levels were similar to the wild-type mice, despite the different circulating androgen and estrogen levels in these mouse models. We thus suggest that in the mice with aberrant P450arom expression during development, the pituitary feedback regulatory system is misprogrammed to allow abnormal circulating steroid levels. This gonadostat regulation is most likely not mediated via ERα, because also in the AROM$^+$/ERKO mice, the LH levels were not different from the WT mice, regardless of the observed increase in E2 concentration.

Despite the normal LH level, the expression of *Star*, *Cyp17a1*, and *Hsd17b3* was significantly lower in the AROM$^+$ mouse testis, suggesting that the down-regulation is mediated via direct estrogen action. Further evidence for this, and the role of ERα in mediating the effects, was obtained by observing a normalized expression for these mRNAs in AROM$^+$ mice crossbred with the ERKO mice but not in those crossbred with the BERKO mice. A direct estrogen effect is also supported by the data showing that in the mice lacking functional androgen receptor, *Star* and *Cyp17a1* are not suppressed (42); neither is the expression of *Star* and *Cyp17a1* changed in cryptorchid mice (43), suggesting that cryptorchidism or low androgen levels in the AROM$^+$ mice do not explain the down-regulation of these steroidogenic enzymes. In contrast, the expression levels of *Insl3*, *Ptgds*, *Vcam1*, and *Hsd17b3* are diminished in the AROM$^+$ mice and in cryptorchid (43) and AR mutant mice (42). It is, therefore, difficult to conclude whether their expression is suppressed by a reduced level of androgens, by an excess of estrogens, or by other mechanisms connected to cryptorchidism. Our results were compared with those of Cederroth and co-workers (11) who exposed pregnant WT, ERKO, and BERKO female mice to pharmacological doses of E2 and diethylstilbestrol and thereafter performed genome-wide expression analysis in fetal testes. Interestingly, four genes (*Insl3*, *Cyp17a1*, *Star*, and *Ren1*) were found to be similarly lower in the studies with fetal (11) and adult-type (present study) Leydig cells after estrogen exposure. Previous studies show the estrogen-dependent regulation of *Cyp17a1* (8) and *Star* (44) genes in Leydig cells, whereas *Ren1* is a novel hormonally regulated gene in the testis. Taken together, our results indicate that estrogens are able to regulate steroidogenesis and Leydig cell function via an ERα-dependent pathway, and this occurs independently of the hypothalamus-pituitary axis.

Our studies also showed that the expression of genes for ECM serine proteases, kallikrein 21, 24, and 27, were significantly lower in the testis of the AROM$^+$ mouse as compared with the WT mouse testis. Reduced levels of kallikreins in the AROM$^+$ testis are most likely due to low androgen levels. Kallikrein 24 has been shown to be androgen but not estrogen regulated *in vitro* (45), and the expression of these three kallikreins has also been found to be reduced in the testis of AR mutant mice (42). In the testis, kallikreins 21, 24, and 27 are exclusively expressed in the Leydig cells, and their expressions are shown to increase during development, being highest at the age of 8 wk (45–47). Thus, they can be considered as adult-type Leydig cell markers. Kallikreins 21 and 24 have trypsin-like and kallikrein 27 chemotrypsin-like activity, and they have been shown to degrade ECM components such as fibronectin, gelatin, and IGF-binding protein-3 (IGFBP-3) (45–47). The improper turnover of ECM due to decreased levels of the kallikreins might thus be one of the mechanisms leading to the development of fibrosis in the AROM$^+$ mouse testis (20). On the other hand, IGFBP-3 is a potent inhibitor of IGF-I-induced T formation (48), and the lack of kallikreins might increase IGFBP-3 concentration and thus suppress T synthesis. Taking into consideration that the adhesion, shape, and proliferation of mouse Leydig cells have been shown to be influenced by ECM *in*

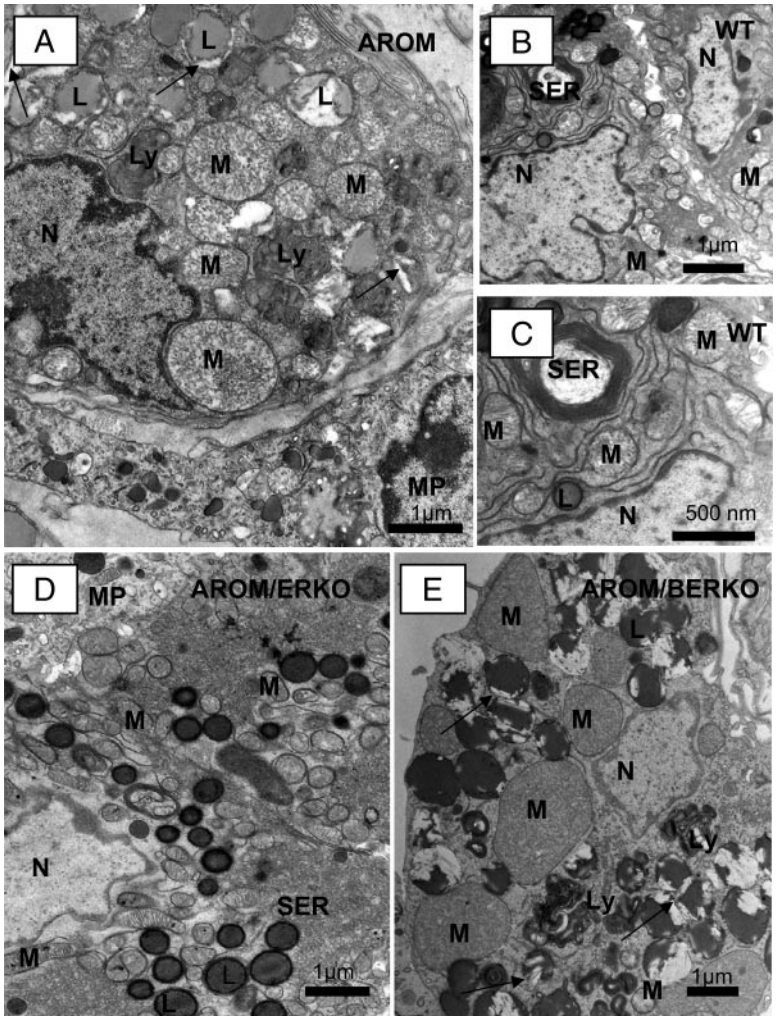

FIG. 5. Ultrastructural studies of the Leydig cells in 3-month-old AROM$^+$ mice (A) showed abundant lipid droplets (L) associated with cholesterol crystals (*arrows*), decreased amount of SER, and strikingly enlarged mitochondria (M) compared with the age-matched WT mice (B and C). The ultrastructure of the Leydig cells of the AROM$^+$ mice crossbred with the ERKO mice (D) resembled that of the WT mice, whereas in the AROM$^+$ mice crossbred with BERKO mice (E), the Leydig cells had similar abnormalities as the AROM$^+$ mice; the mitochondria were enlarged, there was lipid accumulation and cholesterol crystals (*arrows*), and the amount of SER was decreased. Ly, Lysosome, MP, macrophage; N, nucleus. *Bar*, 1 μm (A, B, D, and E) or 500 nm (C).

vitro (49), the low gene expression level of the ECM-degrading kallikreins may have an important role in the pathogenesis of AROM$^+$ testis.

In addition to cholesterol accumulation in the AROM$^+$ Leydig cells, the amount of cholesterol synthesis intermediates was also increased. We therefore postulate that *de novo* cholesterol synthesis is enhanced in the testis of AROM$^+$ mice. In line with these results, there is evidence for a subset of cholesterol biosynthetic genes being directly down-regulated by T (42), whereas no studies are available on estrogen regulation of cholesterol biosynthesis in the testis. Interestingly, accumulation of cholesterol ester in the Leydig cells have been observed in mice deficient in estrogen sulfotransferase (EST), an enzyme that inactivates estrogens (8). Our results, together with studies of EST knockout mice, indicate that estrogens partially regulate de *novo* cholesterol synthesis in the Leydig cells.

Interestingly, the size of the mitochondria in the Leydig cells of 3-month-old AROM$^+$ mice were strikingly increased as compared with WT mice, whereas the structure of mitochondria were morphologically normal. Enlarged mitochondria are connected to insufficient autophagy of the cells (50–53). Normally, mitochondria undergo fusion and fission, and this process has an important role in establishing, maintaining, and remodeling mitochondria (54–56). Formation of giant mitochondria as a consequence of improper steroidogenesis has not been previously reported. Allen and co-workers (15) recently demonstrated that normal mitochondrial membrane potential is required for acute steroid biosynthesis in Leydig cells, and Navratil and co-workers (57) showed that giant mitochondria in rat myoblast cells have lowered membrane potential, leading them to postulate that enlarged mitochondria in AROM$^+$ Leydig cells might also disturb steroidogenesis. The mechanism by which low androgen/high estrogen levels result in the formation of giant mitochondria needs to be studied further. Both ERα and ERβ are localized to the mitochondria of several cell types (18, 58, 59), and they have been shown to induce the expression of mitochondrial DNA-encoded genes as transcription factors (60, 61). Estrogen action in the mitochondria has been shown to increase the capacity for energy production and to decrease the production of reactive oxygen species (17, 18). There are no studies about subcellular localization of ERs in Leydig cells. However, the ultrastructure of the

TABLE 1. Lipid measurements from testis homogenates

	Cholesterol (ng/100 mg testis)	Cholestenol (ng/100 mg testis)	Desmosterol (ng/100 mg testis)	Lathosterol (ng/100 mg testis)	Lanosterol (ng/100 mg testis)
WT					
Free	174.5 ± 3.39	79.8 ± 4.32	3536 ± 90.3	941 ± 22.2	227.2 ± 25.3
Ester	24.38 ± 5.87	26.2 ± 3.83	144 ± 20.7	49.2 ± 4.90	79.4 ± 11.3
AROM$^+$					
Free	204.8 ± 7.45	190 ± 14.43	3770 ± 126	507 ± 40.0[b]	417 ± 90.7
Ester	732.7 ± 98.1[a]	2808 ± 222[b]	2065 ± 101[a]	734 ± 74.4[a]	1163 ± 41.1[a]

Values are given as means ± SEM; n = 5–7. *P* values are between WT and AROM$^+$.

[a] $P \leq 0.01$; [b] $P \leq 0.001$.

Leydig cells, including mitochondrial alterations, was rescued when AROM$^+$ mice were crossbred with the ERKO mice, making it clear that the presence of giant mitochondria is directly or indirectly dependent on ERα.

In conclusion, our study shows that low T in connection with high E2 concentration has several effects on Leydig cell structure and function. In addition to showing an autocrine/paracrine effect of E2 on Leydig cell development and steroidogenesis, we showed that an abnormal androgen/estrogen ratio disturbs cholesterol homeostasis and mitochondrial morphology in mouse Leydig cells. Crossing of AROM$^+$ mice with ERKO or BERKO mice indicated that all the estrogen effects on Leydig cells are directly or indirectly ERα dependent, whereas none of the phenotypes were rescued by crossing with BERKO mice.

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