Aromatase Deficiency Inhibits the Permeability Transition in Mouse Liver Mitochondria

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Lack of estrogens affects male physiology in a number of ways, including severe changes in liver metabolism that result in lipid accumulation and massive hepatic steatosis. Here we investigated whether estrogen deficiency may alter the functionality and permeability properties of liver mitochondria using, as an experimental model, aromatase knockout (ArKO) male mice, which cannot synthesize endogenous estrogens due to a disruption of the Cyp19 gene. Liver mitochondria isolated from ArKO mice displayed increased activity of the mitochondrial respiratory complex IV compared with wild-type mice and were less prone to undergo cyclosporin A-sensitive mitochondrial permeability transition (MPT) induced by calcium loading. The altered permeability properties of the mitochondrial membranes were not due to changes in reactive oxygen species, ATP levels, or mitochondrial membrane potential but were associated with increased content of the phospholipid cardiolipin, structural component of the mitochondrial membranes and regulator of the MPT pore, and with increased mitochondrial protein levels of Bcl-2 and the adenine nucleotide translocator (ANT), regulator and component of the MPT pore, respectively. Real-time RT-PCR demonstrated increased mRNA levels for Bcl-2 and ANT2 but not for the ANT1 isoform in ArKO livers. Supplementation of 17β-estradiol retrieved ArKO mice from massive hepatic steatosis and restored mitochondrial permeability properties, cardiolipin, Bcl-2, and ANT2 levels. Overall, our findings demonstrate an important role of estrogens in the modulation of hepatic mitochondrial function and permeability properties in males and suggest that estrogen deficiency may represent a novel positive regulator of Bcl-2 and ANT2 proteins, two inhibitors of MPT occurrence and powerful antiapoptotic molecules. (Endocrinology 151: 1643–1652, 2010)

The importance of estrogens in the regulation of male physiopathology has only recently been recognized (1). The development of mouse models of estrogen deficiency has played a pivotal role in uncovering the functions of estrogens in men because human clinical cases of estrogen deficiency are extremely rare. We have developed a mouse model of estrogen deficiency by targeted disruption of the aromatase gene (ArKO mice) (2). Aromatase is the enzyme encoded by the Cyp19 gene and is responsible for the biosynthesis of C19 estrogens from C19 steroids. Lack of aromatase in male but not female mice has been shown to cause severe hepatic steatosis as a result of increased triglyceride and cholesterol levels in the liver, a phenotype associated with increased expression of enzymes involved in lipid biosynthesis and decreased activity of enzymes involved in fatty acid β-oxidation (3, 4). Administration of estrogen (E2) significantly reduces the hepatic steatosis and recovers the expression levels and activity of enzymes involved in lipid catabolism (3–5). Consistently three aromatase-deficient men have been reported with hepatic steatosis associated with enlarged liver (6–8), and treatment of one of them with E2 restored

Abbreviations: ANT, Adenine nucleotide translocator; ArKO, aromatase knockout; CsA, cyclosporin A; CypD, cyclophilin D; E2, estrogen; Δψm, mitochondrial membrane potential; MPT, mitochondrial permeability transition; MPTP, MPT pore; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; WT, wild type.
the liver phenotype to normal (7). In addition, both estrogen-deficient men and aromatase-null mice display typical features of the metabolic syndrome, including obesity, insulin resistance, and dyslipidemia (1), demonstrating that estrogens play an important role in regulating some aspects of the male energy metabolism.

Mitochondria are essential intracellular organelles that, in addition to playing a central role in the regulation of the energy metabolism, are also crucial modulators of several aspects of cellular functionality, including cell proliferation, growth, survival, and apoptosis (9–11). A well-known index of mitochondrial dysfunction is represented by changes in the permeability of the mitochondrial membranes, which may affect occurrence of the mitochondrial permeability transition (MPT). MPT is a physiopathological event that results in increased permeability of the inner mitochondrial membrane to solutes with a molecular mass of 1.5 kDa or lower and can disturb the mitochondrial functions in several ways by affecting the cellular bioenergetics, cell survival, and proliferation (12–15). It is now largely accepted that MPT is due to opening of the MPT pore (MPTP) with subsequent release of cations, loss of mitochondrial transmembrane potential, mitochondrial swelling, and apoptosis (12, 16). The MPTP comprises several proteins such as cyclophilin D (CypD) and the ATP/ADP translocator adenine nucleotide translocator [ANT; (15)], and its opening is finely regulated by a plethora of factors, including changes in Ca\(^{2+}\), pH, reactive oxygen species (ROS), and ADP/ATP levels as well as in the expression levels of Bcl-2, an antiapoptotic protein (15, 17). In addition to proteins, changes in cardiolipin, a phospholipid localized almost exclusively in the inner mitochondrial membrane, can regulate the threshold for MPT (18–20). Hormones are also known modulators of the mitochondrial membrane permeability (21, 22); however, as of yet, there is no clear evidence for a potential involvement of estrogens in the modulation of mitochondrial permeability and function in males. This has led us to explore whether estrogen deficiency in ArKO male mice may affect the functionality of liver mitochondria, with a particular interest to the role of estrogens in regulating MPT. The effects of aromatase deficiency on other mitochondrial parameters such as the activity of mitochondrial respiratory complexes, the mitochondrial membrane potential (ΔΨ\(_{m}\)), and the levels of ATP, ROS, Bcl-2, ANT, and cardiolipin have also been analyzed.

Materials and Methods

Mice

Aromatase-deficient mice were created by homologous recombination as described previously (23). The inactivated aromatase gene was maintained on the hybrid 129SvEvTaconic/C57BL6J background. Homozygous null male mice and wild-type (WT) littermates were generated by breeding heterozygous males and females. Offspring were genotyped by PCR as described (24). The animals were maintained in a controlled environment of 12-h light, 12-h dark cycles at an ambient temperature of 22.2°C. Standard irradiated rodent Chow and water were provided ad libitum. Where indicated, 6-month-old mice were treated with 0.5 ng of E\(_2\) in sesame oil vehicle or vehicle alone (n = 3–4 per group) by sc injection three times per week for 8 wk, up to the age of 8 months. No difference was observed between vehicle-treated and untreated mice in all the experiments. All protocols for animal use were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

Cellular subfractionation

Liver mitochondria and cytosol were isolated essentially as described previously (22). Briefly, 8-month- or 8-wk-old fed mice were killed by cervical dislocation after closed cardiac puncture between 0930 and 1030 h. The liver was quickly removed, weighed, and placed in ice-cold sucrose medium (0.25 M sucrose; 5 mM HEPES, pH 7.2; 1 mM EDTA). The liver was minced and homogenized using a Potter-Elvehjem homogenizer with a loose-fitting pestle. Nuclei and cell debris were removed by centrifugation at 500 × g for 10 min at 4°C. The supernatant (homogenate) was centrifuged at 9500 × g for 10 min at 4°C to obtain the mitochondrial (pellet) and the cytosolic fraction (supernatant). The mitochondrial pellet was washed once with sucrose medium and finally diluted to contain approximately 40 mg of mitochondrial protein per milliliter.

Histological analysis

Frozen liver tissues were fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and sectioned for hematoxylin-and-eosin staining.

Lipid and protein content

Approximately 150 mg of tissue (wet weight) were weighed on an analytical balance and pulverized in liquid nitrogen. Lipids were extracted in 30 ml chloroform-methanol (2:1) according to the method of Folch et al. (25), using the protocol optimized for liver tissue by Ametaj et al. (26), and weighed. Total, cytosolic, and mitochondrial protein content was measured using the Bradford’s reagent (Bio-Rad Laboratories, Hercules, CA).

Enzymatic activity of mitochondrial respiratory complexes

The electron transfer activities of complex I/III [nicotinamide adenine dinucleotide (NADH) dehydrogenase/cytochrome bc\(_1\) complex: catalyzes the electron transfer from NADH to ferricytochrome c] and complex II/III (succinate dehydrogenase/cytochrome bc\(_1\) complex: catalyzes the electron transfer from succinate to ferricytochrome c) in isolated mitochondria were assayed by measuring ferricytochrome c reduction (27), using NADH and succinate as substrates, respectively. Briefly, mitochondria (50 μg) were added to an assay mixture (0.5 ml) containing 50 mM potassium phosphate buffer (pH 7.4), 0.3 mM EDTA, 50 μM KCN (Sigma, St. Louis, MO), 150 μM NADH (Sigma) or 20 mM succinate, and 50 μM ferricytochrome c (Sigma). The activity was
determined by measuring the increase in absorbance at 550 nm due to reduction of ferricytochrome c to ferrocytochrome c and confirmed by inhibition with rotenone (5 μM; Sigma) for complex I/III, and antimycin A (30 μM; Sigma) for complex II/III. Specific activity (nanomoles ferricytochrome c reduced per minute per milligram protein) was calculated using the cytochrome c molar extinction coefficient of 21 mM⁻¹·cm⁻¹ (28). The electron transfer activity of complex IV (cytochrome c oxidase: catalyzes the final step of the respiratory chain by transferring electrons from ferrocytochrome c to oxygen) was assayed by measuring ferrocytochrome c oxidation (27). Briefly, mitochondrial (50 μg) were added to an assay mixture (0.5 ml) containing 50 mM phosphate buffer (pH 7.4) and 60 μM ferrocytochrome c. Complex IV activity was determined by measuring the decrease in absorbance at 550 nm after oxidation of ferrocytochrome c to ferrocytochrome c and confirmed by inhibition with KCN (50 μM; Sigma). Specific activity (nanomoles ferrocytochrome c oxidized per minute per milligram protein) was calculated with a molar extinction coefficient of 21 mM⁻¹·cm⁻¹. Where indicated, mitochondria were preincubated for 1 min with 30 μM E₂ (Sigma) or vehicle alone before adding to the reaction mixture.

\[ \Delta \psi_m \]

The membrane potential of intact liver mitochondria was measured following the absorbance decrease at 524 nm of the cationic probe safranine O, using 510 nm wavelength as reference (10, 29). Briefly, freshly isolated mitochondria (0.25 mg protein) were suspended in 1 ml medium containing 0.2 M sucrose, 20 mM HEPES (pH 7.2), 20 mM KCl, 0.38 mM EDTA, 10 mM succinate, added with 20 μM safranine O (Sigma), and the absorbance (524–510 nm) at 25 C was recorded using a spectrophotometer equipped with magnetic stirring and thermostatic control. As control, the ionophore CCCP (1 μM, Sigma) was added at the end of the assay.

**ATP and ROS levels**

ATP levels in total homogenate, cytosol, and isolated mitochondria were determined using a commercially available bioluminescence assay (Sigma), which is based on the reaction of ATP with recombinant firefly luciferase and its substrate luciferin. Briefly, homogenate (20 μg protein), cytosol (20 μg protein), or mitochondria (20 μg protein) were added to a reaction mix containing 25 mM tricine (pH 7.8), 5 mM MgSO₄, 1 mM dithiothreitol, 0.1 mM EDTA, 1.25 μg/ml luciferase, and 0.1 mM luciferin. Luminescence was determined using the SpectraMax 5 luminometer (Molecular Devices, Sunnyvale, CA). The amount of ATP in the experimental samples was calculated from a standard curve generated by using standard dilutions of ATP. Triplicate measurements were performed for each sample.

Mitochondrially generated ROS were determined using dichlorodihydrofluorescein diacetate (Sigma), a membrane-permeable fluorescent dye that is oxidized by ROS into the highly green fluorescent compound dichlorofluorescein, whose fluorescence intensity is directly proportional to ROS production. Briefly, mitochondria (40 μg protein) were incubated in reaction buffer containing 0.25 M sucrose, 5 mM HEPES (pH 7.2), 1 mM EDTA, and 10 μM dichlorodihydrofluorescein diacetate for 20 min at room temperature. Afterward, fluorescence was determined at 488 nm (excitation) and 520 nm (emission). Triplicate measurements were performed for each sample.

**Swelling assay**

To measure changes in the mitochondrial permeability properties, mitochondria (0.35 mg protein per milliliter) were suspended in swelling medium (5 mM succinate-Tris; 10 mM morphinepropanesulfonic acid-Tris; 0.2 M sucrose; 1 mM phosphate-Tris; 2 μM rotenone; and 1 μg/ml oligomycin, pH 7.4) and changes in the absorbance at 540 nm were monitored as described previously (22). Where indicated, 1 μM cyclosporin A (CsA) or 30 μM E₂ (1 min) was added to the medium before mitochondria. CaCl₂ (150 μM) was used as swelling inducer (21).

**Mitochondrial cardiolipin content**

Mitochondrial cardiolipin content was quantitated through an established spectrophotometric assay using nonyl-acridine orange (30). Briefly, mouse liver mitochondria (0.25 mg protein per milliliter) were suspended in 220 mM mannitol; 70 mM sucrose; 10 mM HEPES; and 0.5 mM EDTA, pH 7.4. Aliquots (150 μl) of this suspension were added to increasing amounts of nonyl-acridine orange (1–20 μM), and the final volume was adjusted to 1.5 ml with the suspension buffer. The samples were incubated for 5 min at room temperature and then centrifuged at 35,000 × g for 5 min. Pellets were discarded and the amount of unbound nonyl-acridine orange in the supernatant was measured spectrophotometrically at 495 nm. The number of moles of nonyl-acridine orange per milligram of protein was calculated by subtracting the sample absorbance from that of a standard curve generated using solutions of nonyl-acridine orange (1–20 μM) in the absence of mitochondria. Cardiolipin content was calculated as half of this value considering the 2:1 stoichiometric ratio between nonyl-acridine orange and cardiolipin.

**Immunoblotting analysis**

Total homogenous, mitochondrial, or cytosolic proteins (125 μg) in sucrose medium were added with PBS (pH 7.4) containing 1% Nonidet P-40, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 5 mM sodium pyrophosphate, mixed by vortexing for 5 sec, incubated for 5 min at room temperature, denatured at 70 C for 10 min, and separated on 10% SDS-PAGE followed by transfer on nitrocellulose membrane. Ponceau S staining was routinely performed on each filter to confirm equal loading for all samples. Then membranes were blotted with 1:300 dilution of goat polyclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:300 dilution of goat polyclonal antibody to ANT (Santa Cruz Biotechnology), 10 μg/ml monoclonal antibody to β-tubulin (Sigma), or 1 μg/ml monoclonal anti-CypD antibody (Invitrogen, Carlsbad, CA).

**Real-time RT-PCR**

Total cellular RNA was isolated from frozen tissue samples (0.05 g) using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. One microgram of RNA was used as template for reverse transcription reactions using SuperScript synthesis for first-strand synthesis kit (Invitrogen). Real-time PCR was performed as previously described (10) using 2 μl of reverse transcription reaction. Sequences of forward and reverse primer pairs for ANT1 and ANT2 isoforms were as described by Shabalina et al. (31). Primers for Bcl-2 were as follows: forward, 5’-CAGAAGATCATGCCGTCCTT; reverse, 5’-GTCTACT-
TCTTCCGCAATGC. Amplification of β-actin served as internal control (10).

Statistical analysis

Data are reported as the mean ± SEM of three independent measurements on the samples obtained from four (8-month-old WT and ArKO) or three (8-month-old WT supplemented with vehicle and ArKO supplemented with E2; 8-week-old wild-type and ArKO) different animals for each experimental group. The statistical significance of differences among groups was determined by the one-way ANOVA followed by the Student-Newman-Keuls test. Comparison between independent means was performed using the unpaired Student’s t test.

Results

Liver weight and lipid and protein content

Eight-month-old ArKO male mice developed a significantly heavier liver than WT mice (Fig. 1A) as well as hepatic steatosis characterized by elevated lipid content (8-fold increase compared with WT) and accumulation of lipid droplets (Fig. 1), in agreement with previous results (2–5). However, aromatase deficiency did not affect total or mitochondrial protein content of the liver (Fig. 1A). Supplementation of E2 for 8 wk retrieved ArKO mice from massive hepatic steatosis reducing by more than 70% the lipid content and restoring the liver weight to wild-type levels (Fig. 1).

Aromatase deficiency increases the activity of mitochondrial respiratory complex IV

To investigate whether aromatase deficiency could affect the mitochondrial functionality, we first analyzed the activity of the mitochondrial respiratory chain in isolated liver mitochondria by measuring the ability of the mitochondrial respiratory complexes I-IV to transfer electrons after addition of specific substrates. As shown in Fig. 2A, the activity of complexes I/III (electron transfer from NADH to ferricytochrome c) and II/III (electron transfer from succinate to ferricytochrome c) was comparable in ArKO and WT mice, whereas the activity of complex IV (electron transfer from ferricytochrome c to oxygen) was significantly higher in ArKO mitochondria (P = 0.0008). E2 treatment of ArKO mice resulted in reduction of complex IV activity to levels close to WT (Fig. 2B). To investigate whether E2 had a direct effect on complex IV, mitochondria isolated from WT and ArKO livers were preincubated with E2 or vehicle alone and complex IV activity was measured. As shown in Fig. 2C, in vitro treatment of isolated mitochondria with E2 did not affect com-

![Image](https://academic.oup.com/endo/article-abstract/151/4/1643/2456704)

FIG. 2. Aromatase deficiency increases the activity of mitochondrial respiratory complex IV. A, The specific activity of complexes VIII and I/III in liver mitochondria isolated from WT and ArKO mice (50 μg protein) was measured by following the increase in absorbance at 550 nm of ferricytochrome c, in the presence of NADH and succinate as substrates, respectively. The specific activity of complex IV in isolated mitochondria (50 μg protein) was measured as described in A. C, Mitochondria isolated from WT and ArKO mice supplemented with E2 for 8 wk and WT mice treated with vehicle alone were measured as described in A. C, Mitochondria isolated from WT and ArKO mice were preincubated for 1 min with 30 μM E2 or vehicle alone and then the specific activity of complex IV was measured as described in A. D, Mitochondria (0.25 mg protein) were added with safranine (20 μM) and the decrease in absorbance at 524–510 nm, which is directly proportional to Δψ, was followed spectrophotometrically. As control, the ionophore CCCP (1 μM), which collapses the Δψ, was added at the end of the assay. Representative traces for WT and ArKO liver mitochondria are shown. *, P = 0.0008 vs. WT; **, P = 0.03 vs. vehicle-treated WT mice and P = 0.009 vs. untreated ArKO mice.

![Image](https://academic.oup.com/endo/article-abstract/151/4/1643/2456704)

FIG. 1. Biochemical and histological characteristics of the liver of ArKO mice. A, The weight, lipid content, and total and mitochondrial protein levels were measured in the livers of WT, ArKO, and E2-treated ArKO mice. B, Frozen livers from WT, ArKO and ArKO mice supplemented with E2 were fixed, embedded in paraffin, and sectioned for hematoxylin-and-eosin staining. Accumulation of lipid droplets, evident in the hepatocytes of ArKO mice, is significantly reduced upon E2 supplementation. The arrow indicates the central vein (magnification, ×400).
plex IV activity. Because complex IV works also as a protonic pump of the inner mitochondrial membrane, we then analyzed whether mitochondria isolated from ArKO livers displayed any change in the Δψₘ. As shown in Fig. 2D, higher complex IV activity was not accompanied by changes in Δψₘ, as assessed by similar uptake of the cationic probe safranine in ArKO and WT mitochondria. As control, addition of the uncoupling agent CCCP (a protonophore that discharges the pH gradient destroying the membrane potential) collapsed Δψₘ, in both ArKO and WT liver mitochondria (Fig. 2D). Because mitochondria are the main cellular source of ATP and ROS production (32), both of which are coupled to the activity of the mitochondrial respiratory complexes, we also investigated whether increased complex IV activity could affect ATP and ROS levels in ArKO mice. We found that aromatase deficiency did not alter the ATP levels of isolated liver mitochondria, liver homogenate, and cytosolic fraction or the mitochondrial ROS levels (Supplemental Fig. 1 published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). Overall, these findings show that estrogen deficiency increases the activity of the mitochondrial respiratory complex IV in the liver of ArKO male mice without affecting Δψₘ, ATP, or ROS levels.

**Liver mitochondria from ArKO mice are resistant to Ca²⁺-induced swelling**

To investigate whether aromatase deficiency might affect the mitochondrial permeability properties, the onset of MPT was examined by monitoring Ca²⁺-induced swelling of liver mitochondria isolated from ArKO and WT mice, in presence or absence of CsA, the specific inhibitor for opening of the MPTP (33). Swelling was monitored using a standard spectrophotometric assay as previously described (22). In this assay, an increase in mitochondrial swelling results in a decrease in OD at 540 nm in the mitochondrial suspension. As shown in Fig. 3A, WT liver mitochondria underwent rapid swelling in the presence of Ca²⁺ (ΔA after 10 min: 0.27 ± 0.04), whereas ArKO liver mitochondria were significantly less sensitive to Ca²⁺-induced swelling (ΔA after 10 min: 0.1 ± 0.02; P < 0.001 vs. WT mitochondria). CsA completely prevented mitochondrial swelling in both WT and ArKO mitochondria, confirming that it occurs through opening of the CsA-sensitive MPTP. Supplementation of E₂ to ArKO mice for 8 wk recovered almost completely the ability of liver mitochondria to undergo MPT (ΔA after 10 min: 0.18 ± 0.03; P < 0.005 vs. ArKO mitochondria, P < 0.02 vs. WT mitochondria). However, in vitro preincubation with E₂ of liver mitochondria isolated from ArKO mice did not recover the mitochondrial permeability properties (Fig. 3B). These results demonstrate that estrogen deficiency renders liver mitochondria less sensitive to MPT through a mechanism that does not involve a direct physical interaction of estrogens with the mitochondrial membrane.

To investigate whether changes in the mitochondrial membrane permeability observed in ArKO mice were the result of an aging-related process, onset of MPT was also analyzed in young (8 wk old) ArKO mice. As reported in Table 1, these mice, that exhibit an approximately 2-fold increase in hepatic lipid levels compared with WT mice, displayed reduced sensitivity to MPT, although at lower extent than 8-month-old ArKO mice, indicating that aging does not trigger the changes in mitochondrial membrane permeability in aromatase-deficient mice.

**Estrogen deficiency increases mitochondrial cardiolipin, Bcl-2, and ANT levels**

To get some insights into the mechanism involved in alteration of MPT in the liver of ArKO mice, the levels of cardiolipin, Bcl-2, and ANT, known regulators/components of the MPTP, were analyzed. As shown in Fig. 4, liver mitochondria of ArKO mice exhibited a 1.9-fold increase in the levels of the mitochondrial-specific phospholipid cardiolipin, and E₂ administration was able to restore its content almost completely. A significant increase in cardiolipin levels was also observed in 8-wk-old ARKO mice (Table 1). Bcl-2 and ANT protein levels were mea-
sured in total liver homogenate as well as in the cytosolic and mitochondrial fractions. As shown in Fig. 5A, Bcl-2 and ANT proteins increased in the total homogenate of ArKO liver mice by 161 ± 10 and 163 ± 17%, respectively, as compared with WT mice (P = 0.01). In the mitochondrial fraction, both proteins were dramatically up-regulated in ArKO mice (417 ± 4 and 315 ± 11%, respectively; P < 0.0001), whereas the levels of CypD, a constitutive component of the MPTP, were not affected by aromatase deficiency. Both Bcl-2 and ANT proteins were undetectable in the cytosolic fraction (data not shown). Administration of E2 to ArKO mice reduced Bcl-2 and ANT mitochondrial protein levels to 167 ± 13 and 138 ± 16% of WT, respectively (P < 0.01; Fig. 5B). In addition, young ArKO mice also exhibited a significant increase in mitochondrial Bcl-2 and ANT levels (Table 1), suggesting that estrogen-dependent changes in the liver content of these two proteins occur early during development.

ANT exists in several isoforms, which can exert opposite roles on MPT induction (34–36). To investigate whether aromatase deficiency could modulate Bcl-2 and ANT at the mRNA level and assess which particular ANT isoform was induced by estrogen deficiency, real-time RT-PCR was performed on total RNA extracted from WT and ArKO livers using specific primers for Bcl-2 and the two hepatic isoforms of ANT, ANT1 and ANT2 (37). As shown in Fig. 5C, both Bcl-2 and ANT2 mRNA levels were significantly increased in ArKO livers (178 ± 8 and 165 ± 8%, respectively; P < 0.009), whereas ANT1 mRNA content was unchanged. Supplementation of E2 to ArKO mice retrieved Bcl-2 and ANT2 mRNA levels (Fig. 5C). Overall, these results demonstrate that estrogen de-

### Table 1. Lipid content, mitochondrial permeability, and Bcl-2/ANT protein levels in the liver of 8-wk-old ArKO and WT mice

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<tr>
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<th>WT</th>
<th>ArKO</th>
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<tr>
<td>Liver weight (g)</td>
<td>0.96 ± 0.05</td>
<td>1.02 ± 0.1</td>
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<tr>
<td>Lipid content (mg/g liver)</td>
<td>20.8 ± 1.7</td>
<td>43.1 ± 4.7</td>
</tr>
<tr>
<td>Complex IV activity (nmol/min · mg protein)</td>
<td>258.3 ± 35.6</td>
<td>312.4 ± 23.3</td>
</tr>
<tr>
<td>Mitochondrial swelling (ΔA 540 nm)</td>
<td>0.26 ± 0.05</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Cardiolipin content (nmol/mg protein)</td>
<td>25.4 ± 2.4</td>
<td>32.9 ± 3.2</td>
</tr>
<tr>
<td>Mitochondrial Bcl-2 protein levels (%)</td>
<td>100 ± 9.1</td>
<td>205.3 ± 14</td>
</tr>
<tr>
<td>Mitochondrial ANT protein levels (%)</td>
<td>100 ± 7.2</td>
<td>145.5 ± 6.8</td>
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*Mean ± SEM.

**P = 0.009 compared with WT.

*P = 0.01 compared with WT.

**Changes in mitochondrial OD 10 min after addition of 150 μM CaCl2.

**P = 0.007.
Several factors have been implicated in sensitivity of mitochondria to undergo MPT, including increased ROS production, ATP depletion, changes in $\Delta \psi_m$ (15, 44, 45). We show here that neither ROS nor ATP levels nor $\Delta \psi_m$ are affected by aromatase deficiency, thus excluding these factors from the molecular mechanism of liver MPT resistance mediated by estrogen withdrawal. However, we have found a significant increase of cytochrome c oxidase (complex IV) activity in the liver mitochondria of ArKO mice, which was reduced after E2 supplementation. Decreased activity of complex IV has been associated with a wide range of human pathologies, including stroke, cardioencephalomyopathy, aging, and hepatic failure (46), and it has been correlated with increased sensitization of the cells to apoptotic signals (47). This would suggest that its increase may instead confer a survival advantage to the hepatic cells, either dependently or independently on MPT. In addition, it may provide a kinetic compensation for the reduced availability of energy substrates resulting from impaired fatty acid oxidation in ArKO livers (4). Understanding whether increased complex IV activity may be directly related to resistance to MPT represents an interesting question that remains to be investigated.

Estradiol is a lipophilic molecule that, in addition to being synthesized in the mitochondria, is also transported into these organelles when added exogenously. We show here that treatment of isolated mitochondria with E2 did not affect complex IV activity or interfere with MPT in ArKO or WT liver mitochondria, in agreement with previous reports (48, 49), thus indicating that the estrogen-mediated effects on liver mitochondria permeability properties cannot be ascribed to physical interference/interaction of the estrogen molecules with the mitochondrial membranes. In addition, we found that alterations of the mitochondrial permeability properties are already pronounced in ArKO mice at 8 wk of age, although to a lesser extent than in older (8 months old) mice. Eight-week-old mice exhibited a 2-fold increase in total hepatic lipid content $rs$. 8-fold increase of the older mice as well as increase in the levels of cardiolipin, Bcl-2, and ANT, negative regulators of MPT onset. These findings imply that estrogen deficiency affects male liver physiology and mitochondrial functionality very early in life and that the effects seem to be cumulative with aging but not triggered by it.

It is known that aromatase deficiency alters the maintenance of the hepatic lipid homeostasis; indeed, ArKO males accumulate lipids developing hepatic steatosis spontaneously with aging (2, 4; Fig. 1). We show here that lack of estrogens, besides promoting accumulation of fatty acids, triglycerides, and cholesterol in the male liver (3, 4, 50), affects also the lipid milieu of the hepatic mitochondrial membranes by increasing cardiolipin levels. Cardio-

Discussion

This study presents three new observations: 1) global estrogen deficiency in males affects the liver mitochondrial functionality making liver mitochondria less prone to undergo MPT; 2) aromatase deficiency affects the lipid composition of the mitochondrial membranes in the male liver; and 3) lack of estrogens, specifically estradiol, in males up-regulates the expression of genes implicated in MPT insensitivity and apoptosis resistance.

Liver is a direct target organ of estrogen deficiency in males. Indeed, aromatase deficiency has been shown to cause hepatic steatosis in males accompanied by a plethora of biochemical changes closely associated with the metabolic syndrome (1). Notably, mitochondria play a key role in regulating the whole-cell metabolism; thus, they represent a potential direct target of the estrogen action to influence male liver physiology. Here we show that estrogen deficiency promotes alteration of the liver mitochondrial permeability properties resulting in reduced sensitivity of liver mitochondria to undergo CsA-sensitive MPT. Supplementation of E2 to ArKO mice was able to recover the mitochondrial permeability demonstrating that resistance to MPT in the liver is strictly dependent on lack of estrogens and not due to a developmental and/or an indirect effect of aromatase deficiency. Opening of the CsA-sensitive MPTP has been shown to occur in response to an apoptotic stimulus, causing release of the apoptogenic molecule cytochrome c (15). Consistently, loss of sensitivity to MPT has been associated with resistance of the cells to undergo cell death after an apoptotic stimulus (38); thus, it is conceivable that estrogen deficiency in males may confer a survival advantage to hepatic cells. Of note, estrogen withdrawal after treatment with the anticancer drug tamoxifen, although being able to promote apoptosis of breast cancer cells through inhibition of estrogen receptor action and induction of MPT (reviewed in Ref. (39), also confers a high risk to develop hepatocellular carcinoma (40–42), a side effect that has been associated with inhibition of MPT in liver mitochondria (43), thus suggesting that mitochondrial dysfunction induced by estrogen deficiency may be tissue specific. However, the tamoxifen-related effects on MPT may also be ascribed to estrogen-independent actions on a number of intracellular signaling pathways (39), whereas our animal model of estrogen deficiency rules out any concomitant, estrogen-independent effect, providing the first compelling evidence for a pivotal role of estrogens in modulating sensitivity to MPT in male liver mitochondria.
lipid (diphosphatidylglycerol) is a major structural lipid unique to mitochondria, present predominantly, if not exclusively, in the inner mitochondrial membrane and containing four unsaturated fatty acids and two negative charges. Because of its unique structure among phospholipids, cardiolipin confers fluidity and stability to the inner mitochondrial membrane, and it is also essential as the boundary lipid for the normal functions of proteins of the mitochondrial respiratory chain such as NADH-ubiquinone oxidoreductase, cytochrome c oxidase, F0F1 ATPase, and cytochrome c (51, 52) as well as for the activity of components and regulators of the MPTP, including ANT and members of the Bcl-2 family proteins (53, 54); thus, changes in its content have the potential to significantly affect the mitochondrial functionality. Indeed, loss of mitochondrial cardiolipin occurs with the onset of MPT (55), whereas increased cardiolipin levels have been associated with elevated threshold for MPT (18, 56), consistent with our results showing reduced sensitivity to MPT in ArKO liver mitochondria. The inhibitory effect of cardiolipin on MPT would be due to its strongly negatively charged head groups; indeed, the increased amount of negative charges is thought to nonspecifically bind Ca<sup>2+</sup>, preventing it from acting on protein sites involved in opening of the MPTP. Up-regulation of cardiolipin content could be explained, at least in part, by the increased availability of substrates (fatty acids) resulting from concomitant reduction of fatty acid oxidation (β-oxidation) and enhancement of fatty acid synthesis (3, 4) in the liver of ArKO mice. However, the potential involvement of changes in the activity of cardiolipin synthase, the enzyme responsible for cardiolipin biosynthesis, cannot be excluded and remains to be investigated. Overall, apart from these mechanistic considerations, the finding of increased cardiolipin levels in ArKO liver mitochondria provides the first preliminary evidence of a link between hepatic lipid accumulation and mitochondrial dysfunction owing to estrogen deficiency in males.

Our results show that, besides with cardiolipin, resistance to MPT in ArKO mice is also associated with up-regulation of proteins that modulate opening of the MPTP. In particular, Bcl-2 and ANT, regulator and component of the MPTP, respectively, are increased whereas CypD, structural component of the MPTP and substrate of the MPT’s inhibitor CsA (17), is not affected by aromatase deficiency. Bcl-2 is an oncogenic, antiapoptotic protein located primarily in the outer mitochondrial membrane, mostly at the contact sites with the inner mitochondrial membrane (57, 58), which prevents MPT and cytochrome c release at least in part by blocking voltage-dependent anion channel (a MPT component) and ANT activity (17), although the precise mechanism remains to be characterized. Previous studies have shown that estrogens may have cell-type-specific effects on Bcl-2 expression; for example, E<sub>2</sub> up-regulates Bcl-2 in human macrophages (59), human breast cancer cells (60), and medial amygdala of ovariectomized rats (61), but reduces Bcl-2 levels in human endometrial cancer cells (62). We demonstrate here that aromatase deficiency promotes up-regulation of Bcl-2 mRNA and protein levels in male liver, and, to the best of our knowledge, this is the first time that involvement of estrogen deficiency in up-regulating antiapoptotic proteins in males is reported.

ANT, another molecular target of aromatase deficiency, is a bifunctional protein that catalyzes the exchange of ATP and ADP on the inner mitochondrial membrane and is a component and regulator of the MPTP (17). We found that the cytosolic and mitochondrial ATP content were comparable in ArKO and WT livers, suggesting that the increase in hepatic ANT levels in ArKO mice did not affect ADP/ATP translocation across the mitochondrial membrane. However, ANT induction could regulate MPTP opening. Mouse liver expresses two ANT isoforms, ANT1 and ANT2, homologs of the ANT1 and ANT2 isoforms in human (37). The predominance of ANT1 expression appears to be restricted to tissues unable to regenerate by mitotic division, whereas a prevalent ANT2 transcription occurs in cell types able to proliferate, including liver cells, lymphocytes, and several cancer cell types (35, 63, 64). By real-time RT-PCR experiments, we show that only the ANT2 isoform is induced in the liver of ArKO mice. This is quite intriguing because this specific isoform has been recently identified as an endogenous inhibitor of MPT and thus of apoptosis (35), unlike ANT1, which has proapoptotic function (36). As suggested by Bauer et al. (34), the differential effect of ANT isoforms on MPT onset might be due to specific protein-protein interactions within the permeability transition pore. Besides their opposite role on apoptosis induction, ANT2 but not ANT1 protein is overexpressed in human hepatocellular carcinoma cells compared with normal hepatocytes (35). The ArKO male mice used in this study did not develop macroscopic, palpable liver tumors at the time of the assays (8 wk old and 8 months old); however, it is well known that nonalcoholic fatty liver disease and liver steatohepatitis, common phenotypes in aromatase-deficient men and mice (1, 2, 7, 8), confer a high risk to develop hepatocellular carcinomas (65, 66), and our results strongly support a role for estrogen deficiency in conferring a survival advantage to liver cells, possibly resulting in extended cell lifespan, a condition that may render aromatase-deficient hepatic cells more vulnerable to neoplastic transformation. Histological studies are actually in progress to determine whether ArKO mice develop mi-
crosscopic hepatic tumors at 8 months or whether they may develop tumors at older ages.

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