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Cellular and Molecular Characterization of the Adipose Phenotype of the Aromatase-Deficient Mouse

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Estrogen deficiency in the aromatase knockout (ArKO) mouse leads to the development of obesity by as early as 3 months of age, which is characterized by a marked increase in the weights of gonadal and infrarenal fat pads. Humans with natural mutations of the aromatase gene also develop a metabolic syndrome. In the present study cellular and molecular parameters were investigated in gonadal adipose tissue from 10-wk-old wild-type (WT) and ArKO female mice treated with 17β-estradiol or placebo to identify the basis for the increase in intraabdominal obesity. Stereological examination revealed that adipocytes isolated from ArKO mice were significantly larger and more abundant than adipocytes isolated from WT mice. Upon treatment with estrogen, the volume of these adipocytes was greatly reduced, whereas the reduction in the number of adipocytes was much less pronounced. Transcriptional analysis using real-time PCR revealed concomitant changes with adipocyte volume in the levels of transcripts encoding leptin and lipoprotein lipase, whereas peroxisome proliferator-activated receptor γ levels followed a pattern closer to that of adipocyte number. Little change was observed in levels of transcripts for factors involved in *de novo* fatty acid synthesis, β-oxidation, and lipolysis, suggesting that changes in the uptake of lipids from the circulation are the main mechanisms by which estrogen regulates lipid metabolism in these mice. (*Endocrinology* 144: 1474–1480, 2003)

MODELS OF estrogen insufficiency have revealed new and unexpected roles for estrogens in both males and females. These models include natural mutations of the aromatase gene in men and women (1–3), a natural mutation of estrogen receptor α (ERα) in an individual man (4), as well as the various mouse models of targeted gene disruption, including ERα and ERβ knockouts, ERα/ERβ double-knockout mouse (5–8), and aromatase knockout (ArKO) mice (9–11). It is now apparent from studies of these models that estrogens have many roles in males and females that are not sexually dimorphic and are unrelated to reproduction. These include roles in the maintenance of bone mineralization and brain and vascular function as well as a role in lipid and carbohydrate homeostasis (8, 10, 12–14). We have previously shown that the ArKO mouse develops marked abdominal adiposity with increasing weights of the gonadal and infrarenal fat pads. They also develop hepatic steatosis, particularly the males. This phenotype is progressive with age, and by 1 yr of age the animals display hypercholesterolemia, hypertriglyceridemia, hyperleptinemia, and hyperinsulinemia (10). However, glucose levels are maintained at wild-type levels, indicating that the animals have not progressed to fulminant type II diabetes (10). A similar phenotype has been reported for the ERα KO mouse (15). Ovariectomized rats also develop a phenotype of abdominal adiposity (16). As these animals do not synthesize androgens or estrogens, the phenotype cannot be due simply to the elevated androgens observed in the ArKO mice. Humans with natural mutations of aromatase also develop a metabolic syndrome. Indeed, our most recent patient, a man from Argentina, has insulin resistance accompanied by acanthosis nigricans and hepatic steatosis (17).

Although there was a marked increase in the weight of the intraabdominal adipose depots, in ArKO mice this was not accompanied by a corresponding increase in total body weight. This was because the increase in adiposity was partially offset by a decrease in lean body mass, presumably skeletal muscle (10). Associated with these changes were changes in behavior, in particular a marked decrease in exercise activity as well as a decrease in food intake (10). At this stage it is not clear whether the behavioral changes precede or follow as a consequence of the physiological changes consequent upon estrogen insufficiency.

The present study was designed to explore the cellular and molecular basis for the increase in intraabdominal adiposity. To this end we examined the volume and number of adipocytes in gonadal adipose tissue of female mice at 10 wk of age. We also used real-time PCR to determine the transcript levels of transcription factors and enzymes involved in lipogenesis, lipid oxidation, and lipolysis. These studies have provided insight into the sites of estrogen action to regulate lipid metabolism in gonadal adipose tissue.

Abbreviations: ArKO, Aromatase knockout; CPT1, carnitine palmitoyl transferase 1; ER, estrogen receptor; FAS, fatty acid synthase; HSL, hormone-sensitive lipase; LCAD, long-chain acyl coenzyme A dehydrogenase; LPL, lipoprotein lipase; PGC1, peroxisome proliferator-activated receptor γ coactivator 1; PPARγ, peroxisome proliferator-activated receptor γ; SREBP1c, sterol regulatory element-binding protein 1c; UCP1, uncoupling protein 1; WT, wild-type.

Materials and Methods

Mice

ArKO mice were generated by disruption of the aromatase gene (Cyp19) via insertion of a neomycin-resistant cassette into exon 9 as described by Fisher *et al.* (9). Heterozygous males and females were bred

to produce wild-type (WT) and homozygous-null offspring. Mice were genotyped by PCR as described by Robertson *et al.* (11). Animals were maintained under specific pathogen-free conditions and had unlimited access to drinking water and soy-free mouse chow as previously described (10).

Estrogen replacement

At 7 wk of age, female WT and ArKO mice were implanted with 21-d release 17β-estradiol or placebo sc pellets (0.05 mg 17β-estradiol/pellet; Innovative Research of America, Toledo, OH). After 21 d, gonadal adipose tissue and blood were collected.

Tissue collection

Mice were humanely killed by cervical dislocation. Blood was collected after decapitation and was allowed to clot. Serum was separated and stored at –20 C. Gonadal adipose tissue was removed, and the wet mass was measured. All experiments conformed to the National Health and Medical Research Council (Australia) ethics code of practice.

Tissue preparation

Of the total gonadal adipose tissue collected, 100 mg were snap-frozen in liquid nitrogen and stored at −80 C; 100 mg were immersion-fixed in Bouin's fluid and stored in 70% alcohol at 4 C; the remaining gonadal adipose tissue was processed for adipocyte isolation.

Adipocyte number

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

Adipocyte volume

Bouin's-fixed gonadal adipose tissue was processed in a Histokinette (Leica, Melbourne, Australia), embedded with a random orientation in paraffin, and sliced into 10-μm sections. Sections were stained with hematoxylin, counterstained with eosin, then coverslipped with DPX (BDH, Poole, UK). Adipocyte volume was determined at ×10 magnification as described by Jones *et al.* (10) using CASTGRID version 1.10 (Olympus Corp., New Hyde Park, NY) on an Olympus Corp. BX50 microscope.

RNA extraction and quantification

Total RNA was isolated from 100 mg frozen gonadal adipose tissue using the phenol/chloroform extraction method (Ultraspec RNA, Fisher Biotec, Perth, Australia). RNA was treated with ribonuclease-free deoxyribonuclease (Ambion, Inc., Austin, TX). Total RNA was quantified using a spectrophotometer (DU-530, Beckman, Fullerton, CA), and RNA integrity was confirmed via 1% agarose gel electrophoresis.

Lipid oxidation

Fresh gonadal adipose tissue was digested in fatty acid-free Krebs buffer containing 8.4 ml 5× salt solution (described above), 1.3% $NaHCO_3$, 30% fatty acid-free BSA, 0.2% dextrose, and 240 U/ml collagenase in sterile H_2O. The digest was filtered through gauze to remove debris. The adipocyte layer was collected and diluted to a total volume of 1 ml (in duplicate) in DMEM (Trace Scientific Ltd., Melbourne, Australia) containing 10% fetal calf serum and incubated with 1 μCi [9,10-(N)-^{3}H]palmitic acid (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at 37 C in 5% CO_2 conditions. After the addition of 1 ml trichloroacetic acid, samples were extracted with 4 ml chloroform and further extracted with 2 ml charcoal/dextran solution (5% Norit and 0.5% dextran in sterile H_2O). Aliquots of the aqueous phase were counted employing a liquid scintillation counter. Time-course experiments were carried out to determine the optimal incubation time. The linear range was found between 1 and 5 h of incubation.

Lipogenesis

Fresh gonadal adipose tissue was digested in Krebs buffer (described above). The digest was filtered through gauze to remove debris. The adipocyte layer was collected and diluted to a total volume of 1 ml (in duplicate) in DMEM (Trace Scientific Ltd.) containing 10% fetal calf serum. Cells in medium were incubated with 250 μCi tritiated H_2O (Amersham Pharmacia Biotech) for 6 h at 37 C in 5% CO_2 conditions. The adipocytes were extracted with 5 ml chloroform and washed with an equal volume of sterile H_2O. After evaporation of chloroform, tritium incorporated into lipids was determined with a liquid scintillation counter. Time-course experiments were carried out to determine the optimal incubation time. The linear range was found between 2 and 12 h of incubation.

Glycerol determination

Plasma glycerol was assayed using Triglyceride (GPO-Trinder) Kit 337 (Sigma-Aldrich, St. Louis, MO) for specific determination of the glycerol concentration. Ten microliters of serum from blood were used for each reaction; activity was measured at 540 nm in a spectrophotometer (DU530, Beckman).

RNA expression

Total RNA was isolated from 100 mg frozen gonadal adipose tissue using the phenol/chloroform extraction method (Ultraspec RNA, Fisher Biotec). RNA (1 μg) was reverse transcribed with Expand buffer, 10 mM dithiothreitol, 20 mM deoxynucleotide triphosphate mix, 20 U/reaction ribonuclease I (Roche, Mannheim, Germany), 50 U/reaction Expand (Roche) enzyme, and sterile H_2O to a final volume of 20 μl. cDNA was diluted five times and amplified by real-time PCR in the Lightcycler (Roche) using Fast Start Master SYBR Green I (Roche) and specific oligonucleotide pairs designed to amplify a transcript that spans a minimum of two exons to avoid DNA contamination. Oligonucleotide sequences are shown in Table 1. Real-time PCR data were calculated as a ratio of transcript molecules per microgram of total RNA.

Statistical analysis

Data are expressed as the mean ± SEM. Comparisons between groups were made by univariate analysis (SPSS 10.0, SPSS, Inc., Chicago, IL).

Results

Visual assessment of photomicrographs

Consistent with previous visual examination of gonadal adipose tissue cross-sections (10), Fig. 1A shows that the diameters of the adipocytes from the gonadal adipose tissue of ArKO mice were much greater than those of WT mice. Moreover, estradiol treatment caused a reduction in diameter to values less than those in WT mice.

Adipocyte volume

Stereological examination of adipocyte volume revealed that these changes in adipocyte diameter were indeed indicative of a significant increase in the volume of adipocytes from gonadal adipose tissue of ArKO mice compared with that of WT mice as described by Jones *et al.* (10) and confirmed in Fig. 1B. Figure 1B also demonstrates an associated decrease in adipocyte volume in gonadal adipose tissue of ArKO mice after estrogen treatment.

TABLE 1. Oligonucleotide sequences

Transcript	Sense Primer (5′–3′)	Antisense primer (5′–3′)	Product size (bp)
LPL	AGTAGACTGGTTGTATCGGG	AGCGTCATCAGGAGAAAGG	280
FAS	CACAGATGATGACAGGAGATGG	TCGGAGTGAGGCTGGGTTGAT	205
PGC1	CGCAGCCCTATTCATTGTTC	TCATCCCTCTTGAGCCTTTC	364
SREBP1	ATCGGCGCGGAAGCTGTCGGGGTAG	ACTGTCTTGGTTGTTGATGAGCTGGAGCA	116
Leptin	TCCAGAAAGTCCAGGATGACAC	CACATTTTGGGAAGGCAGG	212
CPT1	ATTCTGTGCGGCCCTTATTGGAT	TTTGCCTGGGATGCGTGTAGTGT	395
LCAD	GCTGCCCTCCTCCCGATGTT	ATGTTTCTCTGCGATGTTGATG	258
UCP1	CACCTTCCCGCTGGACAC	CCCTAGGACACCTTTATACCTAATG	91
HSL	CCTACTGCTGGGCTGTCAA	CCATCTGGCACCCTCACT	142
PPARγ	TTGACAGGAAAGACAACGGA	GAGCAGAGTCACTTGGTCATT	246

	Hybridization probes	
	Probe 1	Probe 2
PPARγ	TTTTTCAAGGGTGCCAGTTTCGATCC Flouro 3′	Red 640 TAGAAGCCGTGCAAGAGATCACAGAGTATG 3′

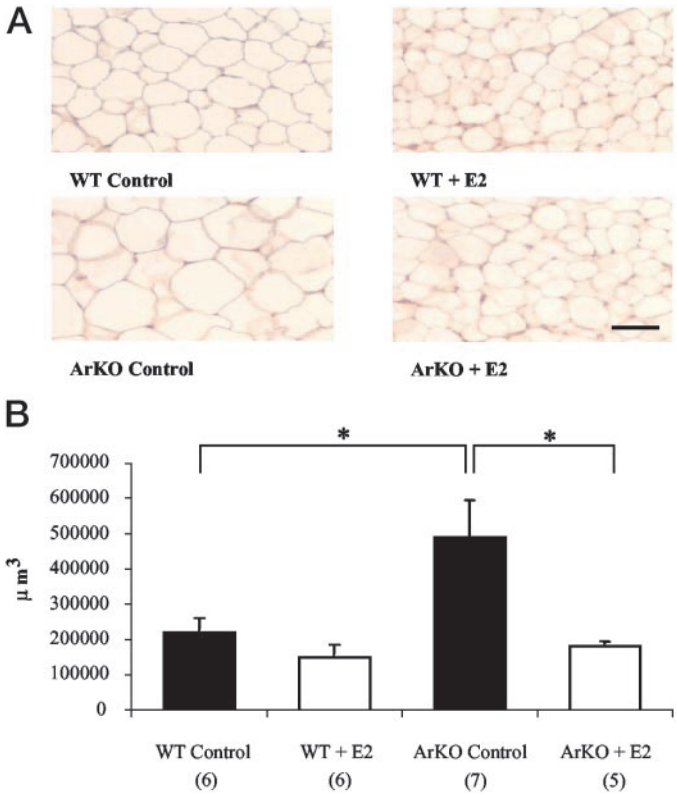

FIG. 1. Adipocyte volume. Gonadal adipose tissue was fixed in Bouin's solution and paraffin embedded. Sections (10 μm) were stained with hematoxylin and eosin. A, Adipocytes were photographed at ×10 magnification. *Bar*, 50 μm. B, Adipocyte volume was measured using CASTGRID version 1.6 (Olympus Corp., Albertslund, Denmark) in a BX50 microscope. Results are presented as the mean ± SEM. *, $P < 0.05$.

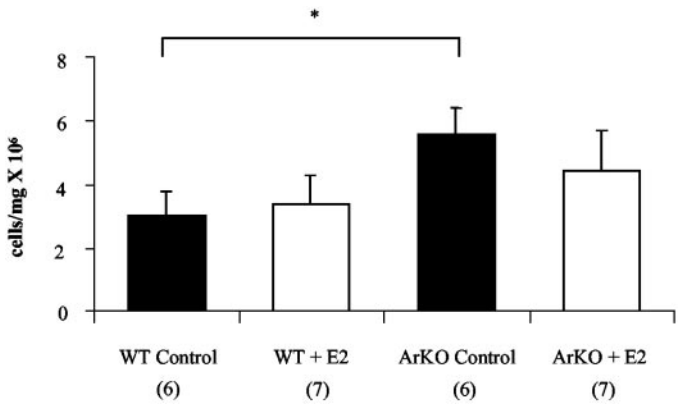

FIG. 2. Adipocyte number. Fresh gonadal adipose tissue was digested in collagenase and stained with methylene blue, and adipocytes were counted with a hemocytometer. Results are presented as the mean ± SEM. *, $P < 0.05$.

Adipocyte number

To ascertain whether there was a corresponding change in adipocyte number, adipocyte-counting experiments were performed (Fig. 2). In accordance with the hyperplasia found in the obese ERα knockout mouse (15), these results showed that gonadal adipose tissue from ArKO mice had a significantly increased number of adipocytes compared with that from WT mice. However, the changes were smaller than those seen in adipocyte volume, and the small decrease after estradiol treatment was not significant. Thus, most of the changes in adipose mass in these experiments are due to changes in adipocyte volume.

De novo lipid synthesis

To investigate changes in *de novo* synthesis of lipid, the incorporation of tritium from [^{3}H]water into total lipid was determined. However, no changes in this incorporation were observed between the WT and ArKO groups (data not shown).

Real-time PCR

To investigate the underlying cellular and molecular mechanisms involved in these changes, real-time PCR was used to quantify the levels of transcripts encoding factors and enzymes involved in lipid metabolism in gonadal adipose tissue. Initially the levels of two RNA species routinely employed as internal standards were determined, namely, 18S RNA and cyclophilin mRNA. In the case of 18S RNA there was a decrease in transcript levels in adipose from ArKO mice, whereas in the case of cyclophilin transcripts there was an increase in the levels of transcripts in ArKO mice relative to those in the other three groups. For these reasons it was decided to express the transcript levels per unit weight of

RNA, as measured by spectroscopy and confirmed by visualization of agarose gel electrophoresis.

Levels of transcripts encoding peroxisome proliferator-activated receptor γ (PPARγ), PPARγ coactivator 1 (PGC1), sterol regulatory element-binding protein 1c (SREBP1c), and fatty acid synthase (FAS)

The levels of transcript for PPARγ were modestly elevated in the gonadal adipose tissue of ArKO mice and were decreased after estradiol administration (Fig. 3A). These results were similar to those obtained for changes in adipocyte cell number. Levels of SREBP1c (Fig. 3B) also appeared to follow those of PPARγ. On the other hand, there was no difference in the levels of transcripts for PGC1 over any of the four conditions (data not shown). The levels of transcripts for FAS were also determined (data not shown), but there was no change in the levels of these transcripts, corresponding to the lack of change in *de novo* lipid synthesis. Based on these data, it would appear that *de novo* fatty acid synthesis is not a factor involved in the changes in adiposity brought about by estrogens.

Lipoprotein lipase (LPL)

We then sought to determine whether lipogenesis from circulating plasma triglycerides might be the pathway most influenced by estrogen. To do this, we measured the levels of transcripts for LPL (Fig. 4A). Transcript levels for LPL were increased in the gonadal adipose tissue of ArKO mice relative to those in WT mice and were suppressed by estradiol administration; these results closely resembled those for adipocyte volume. Thus, it may be that the most important contribution to estrogen-regulated lipid metabolism in gonadal adipose tissue is via regulation of LPL.

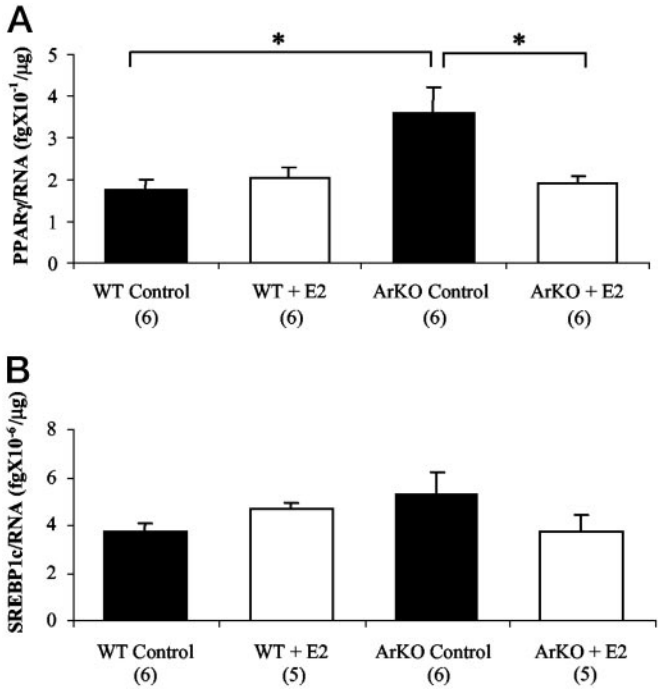

FIG. 3. Differentiation factors. Total RNA was extracted from gonadal adipose tissue using the phenol/chloroform method (Ultraspec RNA, Fisher Biotec) and reverse transcribed with random hexamers. cDNA was diluted five times and amplified by real-time PCR in the Lightcyler (Roche) using specific oligonucleotide pairs. A, PPARγ; B, SREBP1c. Results are presented as the mean ± SEM. *, $P < 0.05$.

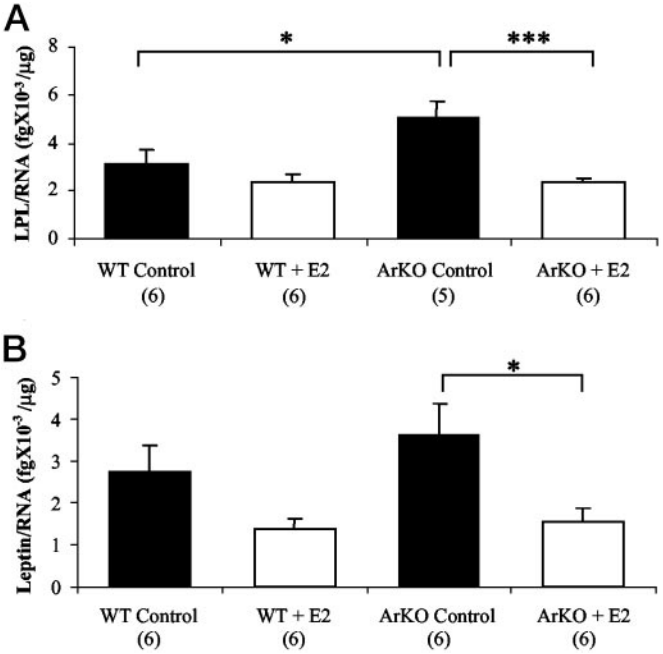

FIG. 4. Lipogenic factors. Total RNA was extracted from gonadal adipose tissue using the phenol/chloroform method (Ultraspec RNA, Fisher Biotec) and reverse transcribed with random hexamers. cDNA was diluted five times and amplified by real-time PCR in the Lightcyler (Roche) using specific oligonucleotide pairs. A, LPL; B, leptin. Results are presented as the mean ± SEM. *, $P < 0.05$; ***, $P < 0.005$.

Leptin

We previously reported that the levels of serum leptin are elevated 2- to 3-fold in ArKO mice compared with those in WT mice (10). Consequently, we determined the levels of leptin transcripts in the gonadal adipose tissue of these mice (Fig. 4B). The levels of leptin transcripts were indeed elevated in the gonadal adipose tissue of ArKO mice and were markedly decreased in both WT and ArKO gonadal adipose tissue upon estradiol administration.

Lipolysis

As treatment with estradiol caused a dramatic reduction in gonadal adipose tissue in ArKO and WT mice (10), we considered it possible that this was an active process and that either lipolysis and/or β-oxidation would be enhanced by estrogen. Blood glycerol levels were determined as a measure of lipolysis (data not shown). No change was seen in the levels in any of the four groups. Transcript levels for hormone-sensitive lipase (HSL) in gonadal adipose tissue similarly showed no differences (data not shown). However, it must be borne in mind that HSL is regulated by stimulation of the catalytic activity via a cAMP-dependent mechanism (18–20). Nevertheless, taken together these results indicate that estrogens do not influence lipolytic activity in the gonadal adipose tissue of these mice.

Factors involved in fatty acid β-oxidation

Fatty acid β-oxidation was measured by the release of tritium from [^{3}H]palmitate into [^{3}H]water. As shown in Fig. 5, estradiol administration elevated β-oxidation rates in WT mice, but had little effect in ArKO gonadal adipose tissue. We also measured the levels of transcripts for long-chain acyl coenzyme A dehydrogenase (LCAD), carnitine palmitoyl transferase 1 (CPT1), and uncoupling protein 1 (UCP1). The levels of transcripts for LCAD followed a pattern similar to that seen in the case of β-oxidation, but there was little change in the levels of transcripts for CPT1 or UCP1. Thus, whereas an increase in β-oxidation might be a contributing factor to the loss of gonadal adipose tissue seen upon estradiol administration to WT mice, surprisingly this does not appear to be the case in the ArKO animals.

Discussion

The development of obesity is associated with both hyperplasia and hypertrophy of cells in the adipose depots (21). Adipocyte number is determined by the replication and differentiation of preadipocytes into mature adipocytes (22). This process is positively regulated by differentiation factors such as PPARγ, PGC1, SREBP1c/adipocyte determination and differentiation 1, and CCAAT/enhancer-binding protein-α and -β (21–25) and is negatively regulated by factors such as TNFα, class I cytokines such as IL-6, IL-11 and oncostatin M as well as by preadipocyte regulatory factor 1 (22, 24, 26–29). The volume of an adipocyte is determined by the balance of three processes occurring within the adipocyte: namely, lipogenesis, lipolysis, and lipid oxidation. Lipogenesis can involve *de novo* synthesis of fatty acids from acetyl coenzyme A and employs acetyl coenzyme A carboxylase (30) and FAS (28, 31, 32). In this case the glycerol component is derived from glycerol-3-phosphate, which, in turn, is formed by reduction of dihydroxyacetone phosphate (28, 33). Adipocyte triglycerides can also be derived from the metabolism of serum lipoproteins such as chylomicrons and very low-density lipoproteins (21, 34, 35), which contain apolipoprotein CII (36, 37). LPL hydrolyzes the ester bonds at the 1,3 positions of the triglycerides, leading to the release of free fatty acids and *sn*2-monoglycerides, which are taken up by the adipocyte and resynthesized into new triglyceride (38). Lipolysis results in the hydrolysis of depot triglyceride to free fatty acids and glycerol via the action of the enzyme HSL (38, 39). These constituents can be released into the bloodstream and taken up by the liver (40) or the fatty acid components can be subjected to the process of mitochondrial β-oxidation, which involves enzymes such as CPT1 (41) and the long (LCAD; Refs. 42 and 43) and medium chain fatty acyl coenzyme A dehydrogenases (44). UCP1 plays an important role in fatty acid oxidation in brown adipose tissue, resulting in dissipation of the released energy as heat (34, 45). Its role together with the other UCPs in white adipose tissue is less clear.

As shown previously, the percentage of body fat as adipose tissue increases in ArKO mice as determined by magnetic resonance imaging (10-wk-old WT females, 4.9%; ArKO females, 18%; Ref. 10), and estrogen replacement (3 wk) in the form of sc implants resulted in a dramatic reduction in the mass of gonadal adipose tissue (10) to values less than those in WT mice.

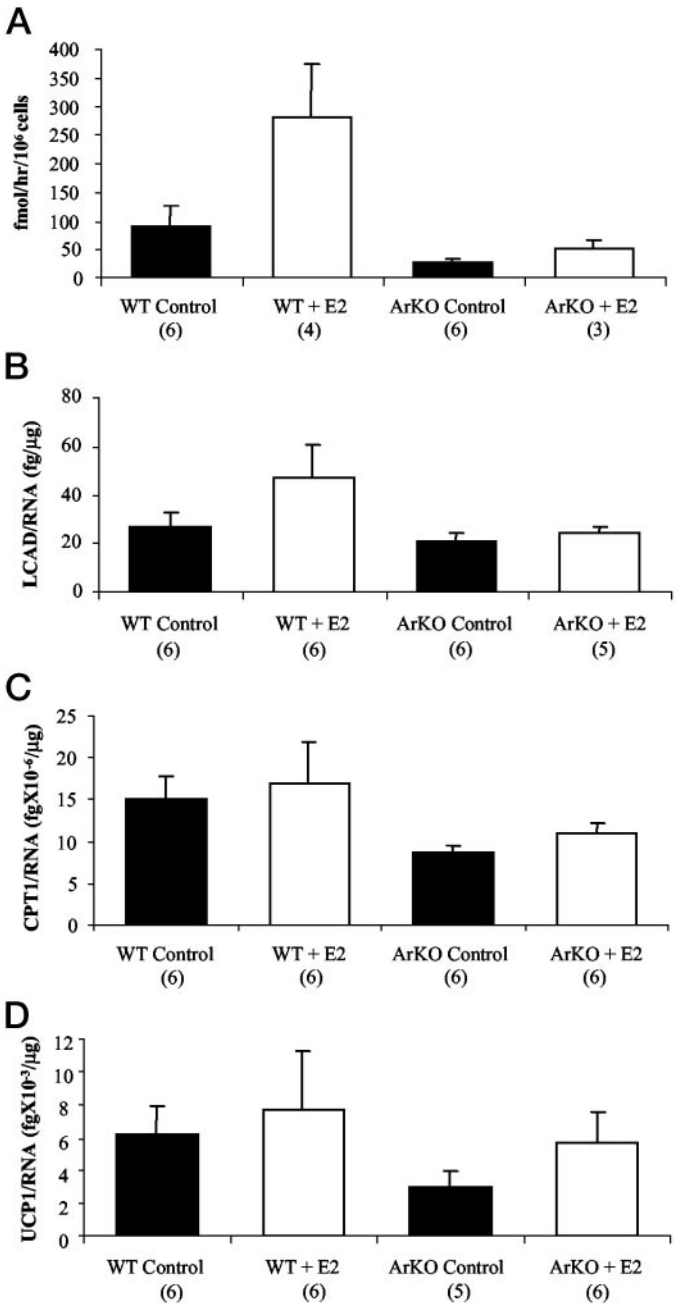

FIG. 5. β-Oxidation. A, Lipid oxidation assay. Gonadal adipose tissue was digested with collagenase. Adipocytes were collected by differential centrifugation and incubated with [^{3}H]palmitate (1 μCi) in a total volume of 1 ml for 1 h. Incorporation of tritium into [^{3}H]water was determined as described in *Materials and Methods*. Total RNA was extracted from gonadal adipose tissue using the phenol/chloroform method (Ultraspec RNA, Fisher Biotec) and reverse transcribed with random hexamers. cDNA was diluted five times and amplified by real-time PCR in the Lightcyler (Roche) using specific oligonucleotide pairs. B, LCAD; C, CPT1; D, UCP1. Results are presented as the mean ± SEM.

The results of the present study indicated that the increase in gonadal adipose tissue of ArKO mice compared with WT

mice is largely a consequence of the increase in volume of preexisting adipocytes, and an increase in adipocyte number plays a lesser role. The increase in the expression of LPL in the fat depots of ArKO mice and its decrease upon estradiol administration appear to be the major transcriptional mechanisms by which estrogen regulates lipid content in these depots. In a recent report it was shown that estrogen acted to suppress the expression of the LPL gene via an ER-mediated mechanism (35, 46). By contrast, the expression of enzymes involved in *de novo* fatty acid synthesis from two-carbon subunits appears to be unaffected by estrogen. As indicated above, PPARγ is a major factor involved in *de novo* fatty acid synthesis as well as in adipocyte differentiation (47, 48), but the levels of its transcripts are only modestly influenced by estrogen withdrawal and administration. PPARγ has been implicated in the regulation of SREBP1c and of fatty acid synthesis (49, 50); however, the levels of transcripts for FAS at least did not appear to follow those of PPARγ. It is interesting that levels of the transcript for PGC1 were also unaffected by any of the four regimens. This implies that the regulation of PGC1 is at the level of its activity rather than its expression.

The marked decrease in gonadal adipose tissue mass after estradiol administration suggests an active process of lipid catabolism. This, however, was not entirely reflected by changes in either the pathway of β-oxidation or the expression of HSL. However, estradiol could increase lipid catabolism by causing changes in the activity of the enzymes involved or in the levels of protein rather than the levels of gene expression. HSL is regulated primarily at the level of catalytic activity by cAMP via mechanisms such as β-adrenergic stimulation (51, 52). One possible mechanism by which estrogen could regulate lipolysis by this pathway is that its actions are largely central rather than peripheral, and the central effects of estradiol result, for example, in an increase in the levels of circulating catecholamines. At this time the relative importance of central *vs.* peripheral actions of estradiol to regulate lipid and carbohydrate metabolism in these mice is unclear, but is the object of ongoing investigations in this laboratory.

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