A selective estrogen receptor α agonist ameliorates hepatic steatosis in the male aromatase knockout mouse

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Abstract

Male aromatase knockout mice (ArKO; an estrogen-deficient model) present with male-specific hepatic steatosis that is reversible upon 17β-estradiol replacement. This study aims to elucidate which estrogen receptor (ER) subtype, ERα or ERβ, is involved in the regulation of triglyceride (TG) homeostasis in the liver. Nine-month-old male ArKO mice were treated with vehicle, ERα- or ERβ-specific agonists via s.c. injection, daily for 6 weeks. Male ArKO mice treated with ERα agonist had normal liver histology and TG contents compared with vehicle-treated ArKO; omental (gonadal) and infra-renal (visceral) fat pad weights were normalized to those of vehicle-treated wild-type (WT). In contrast, ERβ agonist treatment did not result in the similar reversal of these ArKO phenotypes. In vehicle-treated ArKO mice, hepatic transcript expression of fatty acid synthase (*Fasn*) and stearoyl-coenzyme A desaturase 1 (key enzymes in *de novo* FA synthesis) were significantly elevated compared with vehicle-treated WT, but only *Fasn* expression was lowered to WT level after ERα agonist treatment. There were no significant changes in the transcript levels of carnitine palmitoyl transferase 1 (required for transfer of FA residues into the mitochondria for β-oxidation) and sterol regulatory element-binding factor 1c (the upstream regulator of *de novo* FA synthesis). We also confirmed by RT-PCR that only ERα is expressed in the mouse liver. There were no changes in hepatic androgen receptor transcript level across all treatment groups. Our data suggest that estrogens act via ERα to regulate TG homeostasis in the ArKO liver. Since the liver, adipose tissue and arcuate nucleus express mainly ERα, estrogens could regulate hepatic functions via peripheral and central pathways.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) describes a spectrum of progressive liver abnormalities, ranging from benign hepatic steatosis to the more severe non-alcoholic steatohepatitis (NASH) and cirrhosis (Adams & Angulo 2006). NAFLD affects 10–25% of the general population in Western countries (Dixon *et al.* 2001, Angulo 2002), and it is becoming more prevalent due to its close association with the metabolic syndrome (MetS), which is characterized by central obesity, dyslipidemia, and insulin resistance (Qureshi & Abrams 2007). Several epidemiological phenomena suggest a reciprocal link between NAFLD and estrogen action. NASH is more common in men (Clark *et al.* 2003) and in postmenopausal women, suggesting that estrogens have a protective effect from chronic liver diseases in premenopausal women (Ruhl & Everhart 2004, Völzke *et al.* 2007). The administration of tamoxifen (a selective estrogen receptor (ER) modulator commonly prescribed for breast cancer treatment), often results in tamoxifen-induced hepatic steatosis in both obese and non-obese women (Ohnishi *et al.* 2005), and NASH is often observed in men without the presence of obesity, diabetes, or hyperlipidemia (Angulo *et al.* 1999, Yasutake *et al.* 2009). These lines of evidence suggest that an association of low estrogen signaling on hepatic triglyceride (TG) homeostasis independent of increased whole-body adiposity. Moreover, MetS is a common complication in hormonal disorders such as polycystic ovarian syndrome (Sartor & Dickey 2005), as well as congenital aromatase deficiency in men (Maffei *et al.* 2004), in whom estrogen replacement was shown to improve hepatic steatosis and liver function.

Aromatase is the enzyme that synthesizes estrogens from androgens (Gruber *et al.* 2002), and the transcriptional actions of estrogens are mediated through ERα and ERβ (Matthews & Gustafsson 2003). Mouse models of estrogen deficiency develop characteristics of MetS, for example, the aromatase knockout (ArKO) mice exhibit male-specific hepatic steatosis from the age of 3 months accompanied by truncal adiposity, hyperinsulinemia, hyperleptinemia, and dyslipidemia (Jones *et al.* 2000, 2001). The ERα knockout (αERKO) and double ERKO

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mice (DERKO) also developed similar obese phenotypes, but not the ERβ knockout (βERKO; Ohlsson *et al.* 2000), suggesting that estrogen does play an important role in metabolic homeostasis and that ERα is the key receptor involved.

The liver is a key organ for processing and storing lipids in the body, as it is capable of high rates of β-oxidation, lipogenesis, lipolysis, and transport. Hepatic steatosis is the result of excess TG accumulation in the liver, which may be caused by defects in any of the above processes. Previous research from our laboratory showed that hepatic steatosis in the estrogen-deficient male, but not female, ArKO mice was the consequence of molecular changes within the liver, namely elevated transcript levels of fatty acid synthase (*Fasn*, lipogenesis) and adipose differentiation-regulatory protein (*Adrp*, involved in intracellular mobilization and storage of neutral lipids). Both expressions were normalized upon 17β-estradiol (E_2) replacement, coupled with complete reversal of hepatic steatosis (Hewitt *et al.* 2004). The liver has the capacity to store large quantities of TG without causing any ill effects (Novikoff *et al.* 1974, Shimano *et al.* 1996). This is perhaps why fatty liver often remains asymptomatic and undiagnosed until it has progressed to more severe stages such as steatohepatitis and liver fibrosis (James & Day 1998).

This study aims to determine which ER subtype (ERα or ERβ) is involved in the estrogen-mediated reversal of hepatic steatosis by treating male ArKO mice with either ERα- or ERβ-specific agonists. These ER-specific ligands were designed and provided by Bayer Schering Pharma AG (D-13342 Berlin, Germany). The ERα-specific agonist, 3,17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lactone (16α-LE_2), shows 125-fold selectivity for ERα activation, and the ERβ agonist, 8-vinylestra-1,3,5(10)-triene-3,1,7β-diol (8β-VE_2) shows 200-fold selectivity for ERβ activation in a human ER transactivation study (K Prelle, unpublished observations). In ovariectomized rats, 8 days of 16α-LE_2 treatment at doses as low as 0·1 μg/day led to significant uterine hypertrophy (a hallmark of ERα-mediated estrogenic effect), whereas 8β-VE_2 treatment did not have the same effect unless a dosage of 1000-fold was used, highlighting the selectivity of the compounds (Hillisch *et al.* 2004). The fatty liver phenotype in the male ArKO mice becomes fully presented at 1 year of age (Jones *et al.* 2000). Therefore, treatment on male ArKO mice started at 9-month-old and animals were treated till 11 months to observe any reversal effects of the ER agonists from the more severe stage of fatty liver.

Materials and Methods

Animals

The ArKO mice (mixed C57B6 and J129 background) were generated by targeted deletion of exon 9 of the *Cyp19* gene, bred and genotyped as described previously (Fisher *et al.* 1998, Robertson *et al.* 1999). Mice were housed in specific pathogen-free conditions and had unlimited access to drinking water and food. All experimental procedures were approved by the Monash Medical Centre Animal Ethics Committee B. All mice were fed with soy-free mouse chow (Glen Forest stock feeders, Perth, WA, Australia), which contains no soy products as soy contains phytoestrogens that have estrogenic effects (Robertson *et al.* 1999). This diet contains 15% of total calories as fat (0·02% cholesterol), 20% as protein, and 65% as carbohydrate.

Selective ER agonist treatment

Nine-month-old male mice were administered either 3 μg/kg per day of ERα-specific agonist 16α-LE_2 (wild-type (WT) $n=5$; ArKO $n=12$) or 100 μg/kg per day of ERβ-specific agonist 8β-VE_2 (WT $n=6$; ArKO $n=10$) relative to their body weights (BW) via s.c. injection, daily for 6 weeks. Selective ERα (16α-LE_2) and ERβ (8β-VE_2) agonists (Bayer Schering Pharma AG) were first dissolved in methylene chloride (Ajax FineChem, Taren Point, NSW, Australia), and peanut oil was added as the vehicle. The methylene chloride was then evaporated by bubbling with nitrogen gas for 30 min at 37 °C. Control animals were administered oil vehicle (50 μl/animal; WT, $n=10$; ArKO, $n=10$).

The concentrations of ERα and ERβ agonists were chosen based on the original study by Hillisch *et al.* (2004). This study demonstrated that liver parameters were affected by the ERα agonist 16α-LE_2 at the lowest concentration tested (1 μg/day per 220–250 g rat), which is equivalent to that used in this study. There was no effect on these liver parameters from ERβ agonist 8β-VE_2 even at a 100-fold higher concentration (Hillisch *et al.* 2004).

Food intake

Daily food intake (in grams) was measured over 5 days during the final week (week 6) of treatment, where vehicle- and ER agonist-treated mice ($n=5$) were individually housed with food and water *ad libitum*.

Ambulatory activity

To measure ambulatory activity, vehicle- and ER agonist-treated mice ($n=5$) were individually placed in cages equipped with four infrared sensor devices to record their ambulatory movements. The number of times each mouse passed through the sensors was recorded over two continuous days (0900–1700 h) and nights (1700–0900 h) during the final week of treatment. Average counts per day or night were calculated and statistically analyzed.

Tissue collection

BWs were recorded before and after treatment. After 6 weeks of treatment, all mice were killed by carbon dioxide asphyxiation. Blood was collected by cardiac puncture and serum was isolated from whole blood by centrifugation at 2300 ***g*** at 4 °C for

15 min, and stored at −20 °C for TG assay and serum testosterone measurement. Whole liver, omental and infrarenal fat pads referred to as gonadal and visceral fat, respectively, in previous reports (Hewitt *et al.* 2004) were collected and weighed. Small portions of all tissues were fixed in Bouin's solution for histological examination, and the remaining was snap-frozen in liquid nitrogen then stored at −80 °C for expression studies and TG assays. Liver specimens were also embedded in Tissue-Tek (O.C.T (optical cutting temperature) compound Sakura Finetek, Torrance, CA, USA) and stored at −80 °C for frozen sections and histological staining.

Histology

Masson's trichrome staining Tissues were fixed in Bouin's solution for no more than 5 h and stored in 70% ethanol at 4 °C until processing. Liver tissues were embedded random directionally in paraffin and sectioned at 7 μm. Sections were sent to Monash University Histological Laboratory Services for Masson's trichrome staining. Briefly, sections were dewaxed and rehydrated, followed by counterstaining with Weigert's iron hematoxylin (5 min), followed by Masson's trichrome staining, which consisted of Biebrich Scarlet-acid Fuchsin (10 min), 5% tungstophosphoric acid solution (5 min) and aniline blue solution (5 min). Sections were then washed in 1% acetic acid (3 min) and dehydrated with alcohol (2×1 min) and xylene (3×10 s) using standard procedures.

Oil Red O staining Frozen liver specimens embedded in O.C.T (Tissue-Tek Sakura Finetek) were sectioned at 7 μm with the Leica CM 1850 Cryostat (Leica Microsystems, Nussloch GmbH, Nussloch, Germany), and stored at −80 °C until staining. Sections were first fixed in ice-cold 10% formalin (5 min), rinsed in three changes of distilled water and submerged in absolute propylene glycol (5 min; to avoid carrying over of water into Oil Red O (ORO) stain), then stained in 0·5% ORO (Sigma–Aldrich) at 60 °C for 8 min. Sections were rinsed in two changes of distilled water, counterstained with hematoxylin (3 min), followed by rinsing in two changes of distilled water before being mounted with aqueous mounting medium (Dako Corporation, Carpinteria, CA, USA). Lipid droplets were stained red and cell nuclei blue.

Liver TG content

Saponified extracts were prepared from frozen liver samples, and the TG content was quantified by comparing to a glycerol standard curve. First, hepatic TG were extracted by digesting 100–300 mg liver tissues overnight at 55 °C in 350 μl ethanolic potassium hydroxide (2:1 v/v of 100% ethanol and 30% potassium hydroxide; Norris *et al.* 2003). Samples were neutralized with water:ethanol (1:1 v/v; total volume of 1 ml). After centrifugation (8000 ***g***, 5 min), 1 ml of supernatant was collected and further diluted to 1·2 ml with water:ethanol, of which 200 μl proceeded to saponification with 215 μl 1 M $MgCl_2$ by vortexing briefly and incubating at 4 °C for 10 min. Saponified liver extracts (the upper phase) were separated by centrifugation, and the glycerol content was quantified mixing 6 μl extract with 200 μl Free Glycerol Reagent (FGR; Sigma–Aldrich), which would react with free endogenous glycerol to generate a dye with an absorbance at 540 nm. Absorbance (measured using the Wallac 1420 Victor Plate Reader, LabX, Midland, ON, Canada) is directly proportional to free glycerol concentration of the sample. All blanks (6 μl water), glycerol standards (6 μl diluted 1:3; Sigma–Aldrich) and samples were quantified in duplicates.

Serum TG level

Total TG in serum was assayed using the Serum TG Determination Kit (Sigma–Aldrich) according to manufacturer's instructions. Pre-warmed FGR (160 μl) was added to 2 μl undiluted serum sample, and incubated at 37 °C for 5 min, followed by the addition of 40 μl of TG reagent (TGR), and a 5 min incubation at 37 °C, and immediately the initial absorbance (Ai) at 490 nm was recorded (measured using the Wallac 1420 Victor Plate Reader, LabX). The serum total TG content was quantified by adding the TGR (40 μl) to the previous glycerol reactions, and the final absorbance (Af) at 490 nm was recorded after incubation at 37 °C for 5 min. Blanks, standards and samples were quantified in duplicates. Raw Ai and Af values of standards (blank subtracted) were used to generate the glycerol and total TG content standard curves, respectively, against which the cuvette triolein equivalent (CTE; mg/dl) values for the glycerol and TG contents of samples were extrapolated. The glycerol and total TG content (mg/ml) of serum = respective CTE (mg/dl) × (10/30) × (415/200) × 0·012 (dl).

Serum free fatty acid (FFA) levels were measured using the FFA, Half-Micro Test Kit (Roche) according to manufacturer's protocol and adapted for the 96-well format. Briefly, 200 μl of reaction mix A (acyl-CoA synthetase enzyme mixture) was added to 10 μl of serum sample (water as blank) and incubated at room temperature for 10 min to convert any FFA into acyl-CoA. *N*-ethylmaleimide solution was then added to remove excess CoA, before the reading of Ai at 546 nm (Wallac 1420 Victor Plate Reader, LabX). The oxidation reaction of acyl-CoA was then initiated by adding reaction mix B (acyl-CoA-oxidase enzyme mixture) to the previous sample mixture, generating a red dye that was quantitated as above to obtain Af. The final serum FFA was calculated from Af and Ai values as instructed by the manufacturer's protocol.

Serum testosterone level

Serum testosterone levels were measured in duplicates by RIA as described previously (Singh & Handelsman 1996).

Hepatic gene analysis: real-time PCR

Total RNA was isolated from liver tissues using the phenol–chloroform based Ultraspec RNA isolation system (Biotecx,

Houston, TX, USA), DNase treated (Ambion, Austin, TX, USA), and then quantified spectrophotometrically (Radiometer Pacific, Copenhagen, Denmark). RNA quality was assessed by 1% agarose, 1×TBE gel electrophoresis. Reverse transcription PCR was performed by 1 μg total RNA diluted with sterile milliQ water to a total volume of 10 μl. Random primers (50 μg/μl; Promega) were annealed at 72 °C for 5 min, and cDNA was synthesized at 37 °C for 1 h in a 25 μl reaction with 15 μl mastermix containing 5 μl 5×RT buffer, 5·5 μl sterile milliQ water 2·5 μl dNTPs, 1 μl RNAse inhibitor (40 units; Promega), and 1 μl AMV Reverse Transcriptase (20 units; Promega). Gene transcripts were quantified using the LightCycler (Roche) or the Corbett Rotor-gene RG-3000 Real-time PCR machine (Corbett Research, Sydney, NSW, Australia) and specific oligo primer pairs (Table 1), which were optimized to generate single PCR products as confirmed by the expected product size shown by gel electrophoresis and single-peaked melting curves.

All transcript level readings were normalized to cyclophilin transcript expression that is known to be independent of estrogen status (Weisinger *et al.* 1999).

Western blot

Protein lysates were prepared from liver tissue samples (~10 mg) homogenized in 500 μl 1×lysis buffer (25 mM HEPES, 68·5 mM NaCl, 0·5 mM $MgCl_2$, 0·5 mM $CaCl_2$, 5 mM NaF, 1 mM EDTA, and 5 mM Na pyrophosphate with the following added fresh: 1% Igepal (Sigma–Aldrich), 10% glycerol (Sigma–Aldrich) and one tablet of protease inhibitors (Roche) per 10 ml) with a hand-held homogenizer. Homogenates were incubated at 4 °C for 45 min, vortex-mixed occasionally. Lysates were isolated by centrifugation (15 min at 13 200 ***g***, 4 °C) and transferred to new Eppendorf tubes. Protein assay was performed on the lysates using the BCA (bicinchoninic acid) protein kit (Thermo Scientific, Rockford, IL, USA), then stored in aliquots at −80 °C for western analysis.

Liver protein lysates (50 μg in 10 μl 1×lysis buffer) of 11-month-male ArKO and WT mice treated with vehicle, ERα or ERβ agonist, were subjected to western blot analysis. Samples were denatured in 10 μl 2×loading buffer with 200 μM dithiothreitol at 100 °C 10 min before SDS–PAGE (8% acrylamide gel, 80 V for 0·5 h then 150 V for 1 h). The electrophoresed samples were transferred onto a nitrocellulose membrane (Hybond-C Super, Amersham Biosciences) using transblot apparatus overnight (~17 h) at 30 V stirring at 4 °C. Membranes were Ponceau stained for 5 min to visually check the quality of the protein transferred, and gels were coomassie stained to check that transfer was complete. For immunoblotting, membranes were first blocked with 5% skim milk in 1×Tris-based saline tween-20 (TBST) for 1 h at room temperature on a shaker, washed in 1×TBST (3×5 min each), then blotted with rabbit polyclonal anti-FASN primary antibody and rabbit polyclonal anti-ERα primary antibody (1:1000 diluted in 5% BSA in 1×TBST, Novus Biologicals, Littleton, CO, USA) for 1·5 h at room temperature on a shaker. Membranes were then washed and probed with Alexa Fluor goat anti-rabbit 700 secondary antibody (1:5000 diluted in 5% skim milk; Invitrogen) for 1 h at room temperature in the dark on a shaker, washed with 4×5 min then analyzed using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). Membranes were reblocked and

Table 1 Mouse oligo primer sequences for real-time PCR

Gene	Primers[a]	T_A (°C)[b]	Size (bp)[c]	References
Acetyl-CoA carboxylase (*Accα*)	F: TGT TGG GGT TAT TTC AGT GTT GC R: TGT CCA GCC AGC CAG TGT CG	60	236	(Hewitt *et al.* 2004)
Adipose differentiation-related protein (*Adrp*)	F: ACC TTG TGT CCT CCG CTT ATG TCA R: GTT ACG GCA CCT CTG GCA CTG G	67	259	(Hewitt *et al.* 2004)
Androgen receptor (AR)	F: TAT GTG CCA GCA GAA ACG ATT GTA R: CGG TAC TCA TTG AAA ACC AAG TCA	55	541	(Sader *et al.* 2005)
Cyclophilin	F: CTT GGG CCG CGT CTC CTT C R: TGC CGC CAG TGC CAT TAT	60	180	(Boon *et al.* 2005)
Estrogen receptor α (ERα)	F: GCC CTC CCG CCT TCT ACA R: CCC TCC TCG GCG GTC TTT	60	353	Current study
Estrogen receptor β isoforms 1 and 2 (ERβ1/2)	F: TCA TCT TTG CTC CAG ACC TCG T R: GGT AGC CAA GGG GTA CAT AC	58	239 (ERβ2) 180 (ERβ1)	Current study
Fatty acid synthase (*Fasn*)	F: CAC AGA TGA TGA CAG GAG ATG GA R: TCG GAG TGA GGC TGG GTT GAT A	60	205	(Misso *et al.* 2003)
Stearoyl-CoA desaturase 1 (*Scd1*)	F: CAT CAT TCT CAT GGT CCT GCT R: CCC AGT CGT ACA CGT CAT T	55	236	(Biddinger *et al.* 2006)
Carnitine palmitoyl transferase (*Cpt1*)	F: ATT CTG TGC GGC CCT TAT TGG AT R: TTT GCC TGG GAT GCG TGT AGT GT	66	395	(Misso *et al.* 2003)
Sterol regulatory element-binding factor 1c (*Srebf1c*)	F: ATC GGC GCG GAA GCT GTC GGG GTA R: ACT GTC TTG GTT GTT GAT GAG CTG GAG CA	60	116	(Misso *et al.* 2003)

[a]Forward (F) and reverse (R) sense primer sequence in 5′–3′.
[b]Averaged annealing temperature for the primer pair in degrees Celsius.
[c]Expected PCR product size in base pairs (bp).

immunoblotted in the same fashion with mouse anti-β-tubulin primary antibody (1:5000, Millipore/Chemicon, Billerica, MA, USA) and IRDye800CW conjugated goat anti-mouse IgG secondary antibody (1:5000, Rockland, Gilbertsville, PA, USA) as loading controls.

Statistical analysis

All graphs were expressed as mean ± S.D. and fold-change readings were calculated by dividing mean of treatment groups over that of control WT. Significant difference of the means were analyzed between treated WT and ArKO groups by the Mann–Whitney (non-parametric) test and one-way ANOVA, non-parametric Kruskal–Wallis test, with Dunn's multiple comparison *post hoc* test (GraphPad Prism 4 for Windows, GraphPad Software, LaJolla Heights, CA, USA). Differences were deemed significant when $P<0{\cdot}05$.

Results

ERα agonist reduced intra-abdominal fat pad weights

The BW of all treated animals were measured before and after treatment (Fig. 1A). The BW of all groups did not change significantly after 6 weeks of ER agonist treatments compared with before treatment. Previously, we reported that the BW of 6-month-old male ArKO mice were not significantly heavier than WT control (Hewitt *et al.* 2004). In these 9-month-old animals, we did not detect any BW differences between genotypes before or after treatments.

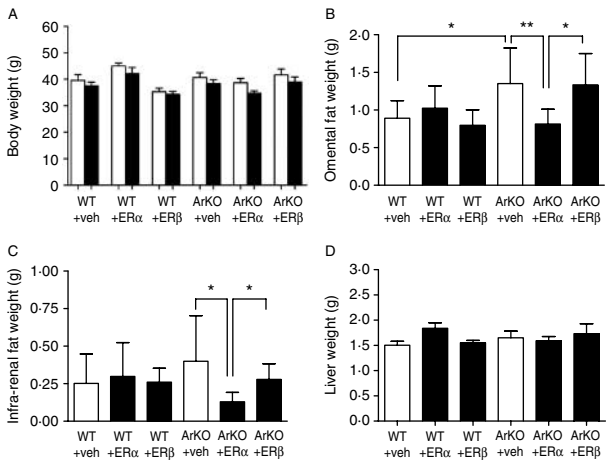

Figure 1 Body and adipose weights. (A) Body weight (BW) before and after treatment was presented in grams. Body weight did not change significantly between treated WT and ArKO male mice in all treatment groups. (B) The omental fat pad weight was significantly greater in the vehicle-treated male ArKO mice compared with WT counterparts. The ERα agonist reduced the weight and normalized it to WT levels. Neither agonist had any significant effects on WT omental fat pad weight. (C) The infra-renal fat pad weight was not significantly different between vehicle-treated ArKO and WT mice, however ERα agonist treatment significantly reduced the weight compared with vehicle-treated ArKO. Neither agonist had any significant effects on WT infra-renal fat pad weight. Results were expressed as mean ± S.D., $n=5$–7 per treatment group. (D) Liver weight was not significantly different among all treatment groups. Significant difference of means were indicated as $^{**}P<0{\cdot}01$ and $^{*}P<0{\cdot}05$.

The wet weights of omental (gonadal) and infra-renal (visceral) fat pads were measured in all treated mice (Fig. 1B). As previously reported, the mean omental fat weight of vehicle-treated ArKO mice was increased to 1·5 times that of control vehicle-treated WT ($^{\star}P=0{\cdot}012$). Significant changes were observed in the ArKO mice after treatments ($^{\star}P=0{\cdot}021$, ANOVA), where ERα-selective agonist significantly reduced this to WT level ($^{\star\star}P<0{\cdot}01$). Treatment with the ERβ-selective agonist did not affect the ArKO omental fat weight.

The infra-renal fat weight (Fig. 1C) of vehicle-treated ArKO mice was not significantly higher compared with WT. The weights were significantly different among treated ArKO mice after 6 weeks ($^{\star\star}P=0{\cdot}0056$, ANOVA), where ERα agonist-treated ArKO mice had dramatically lower fat pad weight compared with that of vehicle-treated ArKO ($^{\star}P<0{\cdot}05$) and of ERβ agonist-treated mice ($^{\star}P<0{\cdot}05$). ERβ agonist treatment did not have any lowering effects in the ArKO mice.

Neither ERα- nor ERβ-selective agonists had any effects on the weight of WT adipose tissues (Fig. 1B and C).

ER agonist treatments did not alter food intake or ambulatory activity

Food intake and ambulatory activities were measured during week 6 of treatment. There were no differences in the daily food intake between any of the treated groups (data not shown). Ambulatory activities during both day- and night-time were not significantly different between any of the groups (data not shown).

ERα agonist ameliorated hepatic steatosis

The mean liver weights were not significantly different between the vehicle-treated ArKO and WT mice, and neither ER agonist had any effect on liver weight (Fig. 1D).

The histology of liver samples were examined using Masson's trichrome staining, (which stains for fibrous tissues; Fig. 2, left column) or ORO staining (which stains for lipid droplets; Fig. 2, right column).

Compared with vehicle-treated WT liver sections (Fig. 2A), the liver of vehicle-treated ArKO was infiltrated by fatty droplets, which appeared as white vacuoles (Fig. 2C). ERα agonist treatment dramatically reduced fat accumulation in the liver thus ameliorating hepatic steatosis in the male ArKO mice (Fig. 2E) whereas ERβ agonist treatment caused a negligible reduction in fatty droplets (Fig. 2G). Masson's trichrome is known to stain fibrous tissues blue, but no fibrous tissues were observed in any of the liver sections, suggesting the absence of hepatic fibrosis in these mice.

To visually compare the amount of fatty deposits, ORO staining was employed to specifically stain the lipid droplets

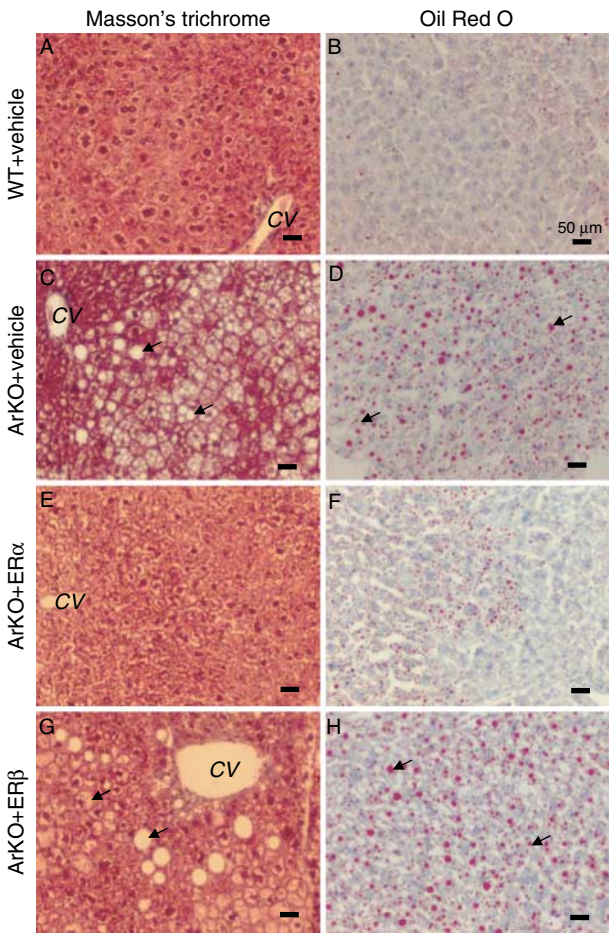

Figure 2 Liver histology. Masson's trichrome stained (left column; staining fibrous tissues blue) and hematoxylin counterstained (purple nuclear stain) liver sections (7 μm), lipid droplets appeared as white vacuoles. Lipid droplets were stained red with Oil Red O (right column). WT + vehicle liver (A and B) had normal liver morphology with very few lipid droplets present. ArKO + vehicle liver (C and D) showed both macrovesicular (ballooning of hepatocytes) and microvesicular (foaming within hepatocytes) types of hepatic steatosis. ERα agonist treatment (E and F) reversed steatosis in the male ArKO liver, which resembled that of WT + vehicle, whereas ERβ agonist treatment (G and H) did not reverse steatosis. Scale bars, 50 μm; C, central vein; arrows indicate the lipid droplets.

red in frozen liver sections. The results were consistent with those revealed by the Masson's trichrome staining, where vehicle-treated ArKO had extensive accumulation of fatty droplets in contrast to the WT liver (Fig. 2B and D), and that ERα agonist, but not ERβ agonist treatment, was able to reduce the extent of lipid accumulation in the male ArKO liver (Fig. 2F and H respectively).

ERα agonist reduced liver TG levels

The TG contents of liver and serum extracts were measured as described in the method and expressed as milligrams of total TG per gram of liver or per milliliter of serum (Fig. 3).

The mean liver TG content (Fig. 3A) of vehicle-treated ArKO mice was significantly elevated compared with that of WT counterparts ($P=0{\cdot}016$, *t*-test). Six weeks of ERα agonist treatments resulted in significant lowering of the TG contents of male ArKO livers (★★$P=0{\cdot}0035$, ANOVA), whereas ERβ agonist treatment did not have a significant effect.

The serum TG, FFA and glycerol levels (Fig. 3B–D respectively) of vehicle-treated ArKO mice were not significantly different compared with vehicle-treated WT. ERβ agonist treatment had no significant effects on all of these serum lipid parameters in both WT and ArKO animals ($P>0{\cdot}05$). ERα agonist treatment significantly reduced serum glycerol in ArKO mice compared with vehicle treatment (Fig. 3D; $P=0{\cdot}048$) but its lowering effects are not significant on serum TG and FFA (Fig. 3B and C).

The mouse liver expresses ERα and not ERβ

The expression of ERs and the androgen receptor (AR) in the adult mouse liver were investigated by real-time PCR (Fig. 4). The adult mouse liver expressed only ERα, and neither ERβ isoforms (ERβ1 and ERβ2) were detected in the male or female, WT or ArKO mice (Fig. 4A). Real-time PCR was used to further quantitate the expression levels of ERα and AR transcripts in the treated livers (Fig. 4B and D

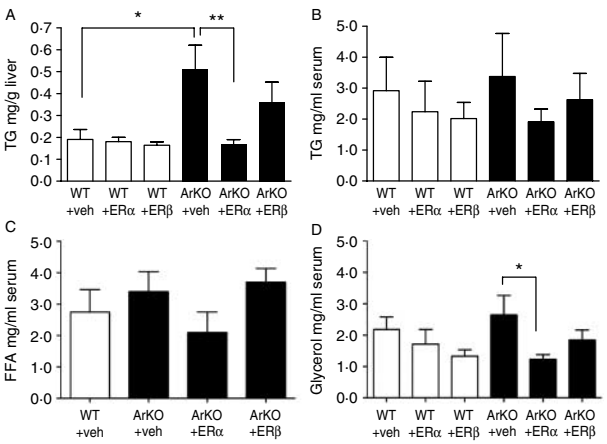

Figure 3 Liver triglyceride contents and serum lipid levels. (A) Liver triglyceride (TG) content was significantly elevated in the male ArKO mice compared with WT (*$P=0{\cdot}016$, Mann–Whitney *U* test); ERα agonist treatment significantly lowered this to WT levels (**$P<0{\cdot}01$, one-way ANOVA), whereas ERβ agonist did not have the same effect. Neither agonist had any significant effects on TG level in WT mice. (B–D) Serum triglyceride (TG), free fatty acid (FFA), and glycerol levels, respectively, were not significantly different between vehicle-treated male ArKO and WT mice. ERβ agonist did not have any significant effect on WT or ArKO (ns; $P>0{\cdot}05$) on these serum parameters. ERα agonist had no significant effects neither on WT mice in terms of serum TG nor on ArKO in terms of serum TG and FFA, however it significantly lowered serum glycerol level of ArKO compared with vehicle-treated ArKO ($P=0{\cdot}048$). Results were expressed as mean ± S.D., $n=5$–7 per treatment group.

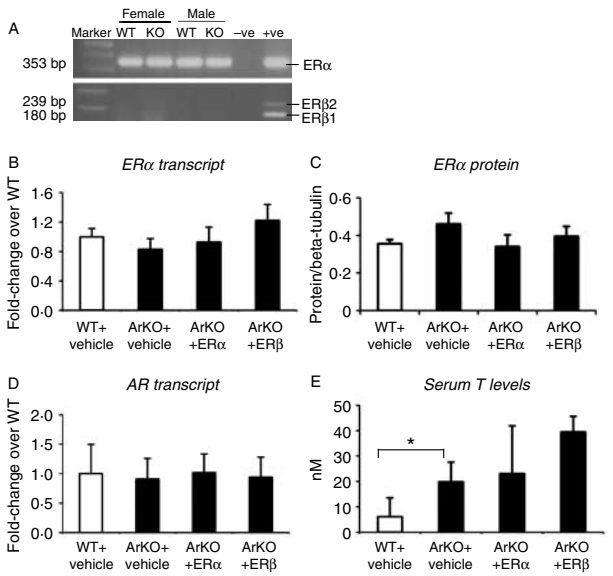

Figure 4 Liver estrogen receptor (ER) and androgen receptor (AR) transcripts, and serum testosterone level. (A) Gel electrophoresis of real-time PCR products indicated that ERα is expressed in livers of both male and female, WT and ArKO mice (top panel); no ERβ1 and ERβ2 transcripts were detected in any of the liver samples. WT mouse ovary cDNA and no template samples were used as a positive control (+ve) and negative control (−ve) respectively. No significant differences were detected in the hepatic (B) ERα transcript and (C) protein level, nor in (D) AR between male WT+vehicle and ArKO+vehicle livers, and neither agonist had any effect on the ERα or AR expressions. Real-time PCR results were normalized against the housekeeping gene cyclophilin, western blot results were normalized against β-tubulin, expressed as mean±S.D. and fold-change over WT+vehicle, $n=5$–7 (ERα) or 8–12 (AR) per treatment group. (E) Serum testosterone (T) level was measured by RIA as described previously (methods). Serum T was over three times higher in vehicle-treated ArKO compared with vehicle-treated WT. ArKO serum T level was not affected in by ERα or ERβ agonist treatments. Results were expressed as mean±S.D., $n=4$–7 per treatment group. Significant difference of means were indicated *$P<0{\cdot}05$.

respectively). The protein levels of ERα were measured by western blot analysis (Fig. 4C). There were no significant changes in the hepatic expression levels of ERα or AR between vehicle-treated WT and ArKO male mice (Fig. 4B–D respectively). Neither ERα nor ERβ agonist had any effects on ERα or AR expression in the treated ArKO mice. Serum testosterone level was significantly elevated (approximately threefold) in the male vehicle-treatment ArKO mice compared with WT ($P=0{\cdot}032$, Fig. 4E), but neither ERα nor ERβ agonist treatment could lower the male ArKO serum T levels to WT levels.

ERα agonist reduced expression of key enzymes involved in de novo fatty acid (FA) synthesis in the male ArKO liver

Transcriptional changes in key genes involved in FA metabolism may underlie the mechanism(s) behind the amelioration of hepatic steatosis by estrogen in the ArKO male mice. This was investigated by analyzing the transcript levels of key hepatic genes using real-time PCR (Fig. 5). Since neither ER agonist had any significant effects on WT BW, omental and infra-renal fat weights and liver phenotype, we did not pursue analysis of liver gene expression in these mice.

Our results indicated that the transcript level of *Fasn*, in vehicle-treated male ArKO liver was increased to 1·8 times that of control WT (⋆$P=0{\cdot}028$; Fig. 5A). Liver Fasn expression is different among the male ArKO mice after 6 weeks of treatments (⋆⋆$P=0{\cdot}0015$, ANOVA), where ERα agonist treatment dramatically reduced the expression level and normalized it to WT level (versus vehicle-treated ArKO ⋆⋆⋆$P<0{\cdot}001$), whereas ERβ agonist did not have the same lowering effect, despite a slight reduction that was not of statistical significance ($P=0{\cdot}068$).

Stearoyl-coenzyme A desaturase 1 (Scd1) transcript level was also significantly elevated in the vehicle-treated ArKO compared with WT ($P<0{\cdot}05$), but neither ER agonist had any significant effects on its expression in the ArKO liver (Fig. 5B).

Acetyl-CoA carboxylase α acts upstream of *Fasn* in FA synthesis by producing malonyl-CoA as the substrate for *Fasn* (Wakil *et al.* 1983). However, no differences in its transcript level were detected between treated or control groups (Fig. 5C).

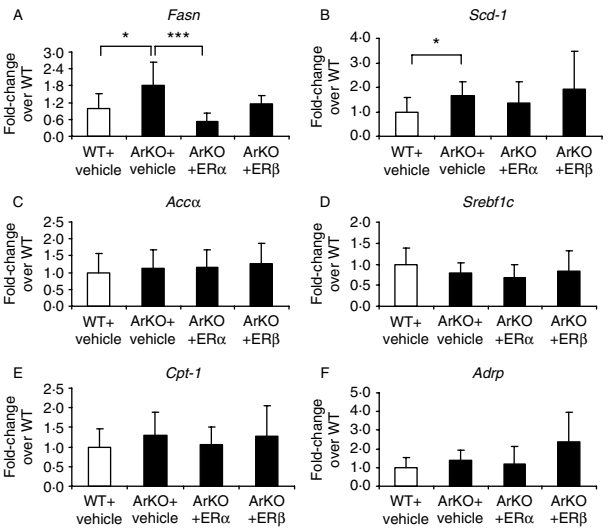

Figure 5 Hepatic expressions of genes involved in triglyceride metabolism. Transcript levels were quantified by real-time PCR. (A) *Fasn* expression was significantly elevated in the livers of male ArKO+vehicle group and ERα agonist treatment normalized it to WT+vehicle level. (B) *Scd1* expression was also significantly elevated in ArKO+vehicle group but neither ER agonist had any significant effects. (C–F) No significant changes were observed in the hepatic expressions of *Accα, Srebf1c, Cpt1*, and *Adrp*. Results were normalized to the housekeeping gene cyclophilin, and were expressed as mean±S.D. and fold-change over WT+vehicle, $n=7$–11 per treatment group. Significant difference of means were indicated *$P<0{\cdot}05$ and ***$P<0{\cdot}001$.

To investigate the upstream stimulation of *Fasn* transcription, the expression of sterol regulatory element-binding factor 1c (*Srebf1c*) was studied. SREBF1c is a master regulator of *de novo* FA synthetic enzymes (Horton *et al.* 2002). However, the hepatic expression level of *Srebf1c* was not different between vehicle-treated WT and ArKO mice (Fig. 5D), and neither agonist had any significant effects on its expression in the male ArKO mice.

Carnitine palmitoyl transferase 1 (CPT1) promotes mitochondrial FA β-oxidation by regulating FA transport into the mitochondria, and its activity is allosterically inhibited by malonyl-CoA (McGarry *et al.* 1978). We did not detect any significant change in liver *Cpt1* transcript levels between any of the treatment groups (Fig. 5E).

ADRP is a lipid droplet surface protein within hepatocytes, whose expression was found elevated in the male ArKO liver (Hewitt *et al.* 2004) as well as in human steatotic livers (Motomura *et al.* 2006). However, our current data did not reveal any significant difference in its transcription in the male ArKO (Fig. 5F).

Western blotting showed that male ArKO mice have an increase in hepatic FASN protein level compared with vehicle-treated WT that did not reach statistical significance ($P=0{\cdot}083$; Fig. 6). ERα agonist treatment significantly reduced hepatic FASN protein level compared with vehicle-treated ArKO ($P<0{\cdot}05$; Fig. 6) whereas the ERβ agonist did not have a significant effect.

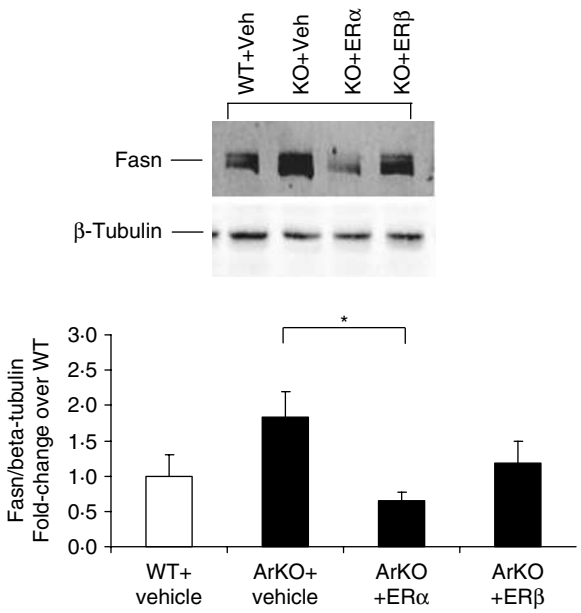

Figure 6 Hepatic FASN protein expression. Liver lysates from 11-month-old male vehicle-treated WT and ArKO, ERα agonist- and ERβ agonist-treated ArKO were subjected to western blot analyses. Hepatic FASN protein level of vehicle-treated ArKO mice was not significantly higher than that in WT ($P=0{\cdot}083$, Student's *t*-test), but ERα agonist treatment significantly reduced FASN expression to WT level compared with vehicle-treated ArKO ($P<0{\cdot}05$, ANOVA) whereas ERβ agonist did not. Results were normalized to β-tubulin and expressed as mean±S.D., then as fold-change over WT, $n=8$–10 per treatment group. Significant difference of means were indicated $^*P<0{\cdot}05$.

Discussion

This study investigated the phenotype of hepatic steatosis in older, estrogen-deficient male ArKO mice, and indicates that this is a consequence, in part, of continual increased fatty acid synthesis at the liver. Furthermore, it revealed that estrogen ameliorates hepatic steatosis predominantly via ERα-mediated pathways. Our laboratory has previously reported severe hepatic steatosis in males but not females, and a significant increase in intra-abdominal adiposity in both sexes by 6 months of age compared with WT controls, all of which were normalized upon estrogen replacement (Hewitt *et al.* 2004). Real-time RT-PCR analysis previously reported notable upregulation of hepatic *Fasn* and *Adrp* gene expression, which was speculated to be the main cause of hepatic steatosis in the male ArKO mice. These data together with our current results, suggest that estrogen replacement can reverse excess adiposity and hepatic steatosis in the male ArKO mice by acting through ERα.

ERα-mediated pathways at the liver

Our data and those of others (Couse *et al.* 1997) show that the mature mouse liver expresses only ERα and not ERβ. The expression level of ERα appears identical between male and female, WT and ArKO livers; therefore, liver expression of ERα or AR does not explain the sexually dimorphic liver phenotype in the ArKO mice. Estrogen deficiency is also accompanied by high androgen levels, which have been associated with increased visceral adiposity in the female ArKO mice (McInnes *et al.* 2006) and not with fatty liver since female ArKO mice do not develop fatty liver (Hewitt *et al.* 2004). Androgens are also linked to GH-induced male-specific liver gene expression that may affect liver metabolism (Thompson & Lucier 1983). Vehicle-treated ArKO mice were shown to have elevated testosterone levels, but the reversal of fatty liver by ERα agonist was not associated with a decrease in testosterone levels. Hence, the loss of hepatic fat was not due to the lowering effect of serum testosterone and likewise, hepatic steatosis in the male ArKO may not be due to elevated testosterone. Indeed, female ArKO mice, which have normal, non-steatotic livers, also have elevated serum testosterone levels (Fisher *et al.* 1998).

Our data revealed that selective ERα activation significantly normalized *Fasn* expression (at transcript and protein levels), which was significantly upregulated in the steatotic livers of untreated male ArKO mice as they approached 1 year of age. FASN enzyme catalyses the synthesis of palmitate from malonyl-CoA, and palmitate is the precursor to all other fatty acids via subsequent elongation and desaturation reactions toward TG synthesis (Smith 1994). ERβ agonist treatment

had no significant effect on hepatic *Fasn* at transcript and protein levels. These current observations are in line with those reported by another study, whereby the antagonistic action of tamoxifen was found to induce *de novo* fatty acid synthesis, in particular via the upregulation of hepatic *Fasn* transcription in mice with hepatic steatosis (Cole *et al.* 2010). A recent study also observed an ER-dependent down-regulation of *Fasn* transcription in ovariectomized mice followed by estrogen replacement (Della Torre *et al.* 2011).

SCD1 enzyme further processes the synthesized palmitate by desaturation to produce palmitoleic acid (Ntambi & Miyazaki 2003). Nemoto *et al.* (2000) reported that several fatty acids were increased in the male ArKO liver, with palmitoleic and eicosatrienoic acids having the greatest folds of increase. Consistent with this, we observed an increase in *Scd1* expression in the male ArKO liver compared with WT. However, neither ER agonist had any significant effects on *Scd1* transcription, therefore the lowering effect of ERα activation was only limited to the FASN-mediated step of *de novo* FA synthesis.

SREBP1c enzyme is known to promote *de novo* FA synthesis by upregulating the expression of lipogenic genes such as *Fasn* and *Scd1* in the liver (Edwards *et al.* 2000). Transgenic mice of Srebf1c (different nomenclature for mouse but referring to the same protein) developed fatty liver with increased hepatic TG content (Shimano *et al.* 1997). The elevated levels of *Fasn* and *Scd1* of the male ArKO would suggest an elevation in *Srebf1c* expression in the fatty liver. However, we did not observe such increase in *Srebf1c* transcription in the male ArKO mouse liver. *Srebf1c* transcription is known to be upregulated in response to insulin signaling (Shimomura *et al.* 1999), and likely insulin signaling may be compromised or impaired in the ArKO liver, thus prohibiting insulin-mediated upregulation of *Srepf1c.* This leads us to speculate that Srebf1c activity may have been affected posttranslationally by estrogen deficiency. The action of Srebf1c is initiated by the proteolytic release of its N-terminus, which is then translocated into the nucleus to activate transcription of target genes (Brown & Goldstein 1997). The lack of estrogen may have promoted the occurrence of this process, thus enhancing FA synthesis. As a precedent, thyroid hormones have been demonstrated to promote the proteolytic processing of Srebf1c and the subsequent increase in *de novo* FA synthesis in rat livers (Ståhlberg *et al.* 2005). Further experiments are in progress to determine whether proteolytic activation of Srebf1c is indeed increased in the ArKO liver.

We also examined the expression of Cpt1, a rate-limiting step in fatty acid β-oxidation, and found no change in its transcript level. This is in accordance with our previous report that there were neither changes in the expression of enzymes involved in the fatty acid oxidation pathway nor in palmitate oxidation, in ArKO versus WT livers (Hewitt *et al.* 2004). However, this is in contrast to the findings of Nemoto *et al.* (2000), who reported impaired FA β-oxidation in their male ArKO mice. We conclude that in the ArKO mice that we have generated, differences in fatty acid oxidation do not have a significant role to play in the sexually dimorphic accumulation of hepatic TG.

In contrast to previous reports in humans and 6-month-old mice (Hewitt *et al.* 2004, Motomura *et al.* 2006), the hepatic expression of *Adrp* was not markedly increased in the steatotic vehicle-treated ArKO compared with control WT used in this study, and neither ER agonist had any significant effects on its expression. A possible explanation is that *Adrp* expression began to decrease at full presentation of hepatic steatosis, since *Adrp* mRNA is only expressed very early during differentiation in adipose tissues, i.e. increases by day 1 but decreases by day 4 of differentiation (Brasaemle *et al.* 1997). This may explain why *Adrp* transcript was found increased at the early stage of hepatic steatosis at 6 months (Hewitt *et al.* 2004) but not in the current 11-month-old male mice.

Serum TG levels in ER agonist-treated WT and ArKO mice showed similar, although not statistically significant, patterns (i.e. increased in male ArKO and a trend to decrease after ER agonist treatments) to those of liver TG contents, presumably reflecting the upregulation of synthesis and output from the liver. Previous studies have also found no significant differences in serum TG levels between both female and male WT and ArKO mice, except until 1 year of age in male ArKO mice (Jones *et al.* 2000).

ERα-mediated pathways: peripheral effects

In mice and humans, ERα is the predominant ER expressed in adipose tissues (Lundholm *et al.* 2004), and our previous studies are consistent with direct actions of E_2 via ERα on the adipose depots (Misso *et al.* 2003, Hewitt *et al.* 2004, McInnes *et al.* 2006). Our data show that the intra-abdominal adiposity (omental and infra-renal fat depots) was reduced by the selective activation of ERα in the male ArKO mice. Obesity is one of the main risk factor for fatty liver, while physical exercise and weight loss remain the most common strategies in treating patients with NAFLD (Adams & Angulo 2006). It is believed that a reduction in fat depots (increased oxidation and/or decreased *de novo* FA synthesis) can result in decreased hepatic fat deposition due to diminished FFA release from adipose tissues (Bradbury 2006). Therefore, it is possible that the ERα-mediated reduction of hepatic steatosis may be regulated in part by reducing intra-abdominal adiposity, and thus decreasing FFA transport to the liver. Indeed, in preliminary experiments both serum glycerol and FFA levels in ERα agonist-treated male ArKO mice appeared to be lower than those of vehicle-treated ArKO mice. Indeed, our experiments showed that both serum glycerol and FFA levels in ERα agonist-treated male ArKO mice appeared to be lower, especially the serum glycerol level that was significantly lowered with ERα agonist compared with vehicle-treated ArKO mice.

ERα-mediated pathways: central effects

The brain, in particular the arcuate nucleus in the hypothalamus, is known to control feeding behaviors and energy expenditure. ERα is the major ER subtype expressed in the mouse arcuate nucleus (Mitra *et al.* 2003). Therefore, it is possible that lack of central ERα activation at the arcuate nucleus also plays a role in the sexually dimorphic development of hepatic steatosis in the estrogen-deficient ArKO mice.

However, we did not observe any changes in food intake in both vehicle- and ER agonists-treated ArKO mice compared with WT. Hence, the decrease in truncal adiposity and reversal of hepatic steatosis after ERα treatment was not the consequence of changes in feeding behavior of the male ArKO mice. A previous study also reported that ArKO food intake was unaltered by estrogen deficiency (Jones *et al.* 2000). Both ERα-specific agonist 16α-LE_2 and ERβ-specific agonist 8β-VE_2 have been shown to have the ability to cross the blood brain barrier in our parallel study (Hill *et al.* 2007*a*), which reported that either 16α-LE_2 or 8β-VE_2 treatments could reduce in apoptosis at specific regions of hypothalami of these same groups of male ArKO mice. However, at this point we do not know why our dosage of 16α-LE_2 did not affect food intake; one possible explanation may be the dosages of selective ERα agonist PPT used in other studies were much higher than our agonist dosage used: ~1800 μg/kg per day for rats (Roesch 2006) and 4000 μg/kg per day for mice (Thammacharoen *et al.* 2009) versus our dosage of 3 μg/kg per day.

While ambulatory activity was previously shown to be reduced in the younger male ArKO mice (Hill *et al.* 2007*b*), the reduction of intra-abdominal and hepatic adiposities was not due to the normalization of energy expenditure, since neither current ER agonists nor E_2 treatment in previous studies (Hill *et al.* 2007*b*) enhanced ambulatory activity in ArKO mice.

Nevertheless, the explanation of the sexually dimorphic phenotype of the hepatic steatosis in the ArKO mouse may lie in the sexually dimorphic nature of the arcuate nucleus. We have previously established that estrogen deficiency does result in apoptosis of the arcuate nucleus in male, but not female, ArKO mice (Hill *et al.* 2004). Furthermore, selective activation of ERα rescued this apoptotic phenotype (Hill *et al.* 2007*a*). Taken together, our data suggest that signals from the hypothalamus influence the liver in a sexually dimorphic fashion. GH signaling provides such stimulus, and results in sexually dimorphic imprinting of a number of liver enzymes in rodents (Waxman & O'Connor 2006). Whether altered patterns of GH signaling are responsible for the male-specific hepatic steatosis observed in the ArKO mice will be the subject of future investigations. To ascertain whether the ArKO hepatic steatosis is reduced by a direct activation of ERα by 16α-LE_2, an ERα-selective antagonist (i.e. ICI 1 82 780) will be administered concurrently in future studies.

In summary, our data demonstrated that selective ERα activation can ameliorate hepatic steatosis in the male ArKO mice. This ERα-mediated reversal is due, in part, to changes in hepatic expression of *Fasn*.

Declaration of interest

K P is an employee of Bayer Schering Pharma AG; remaining authors of this work have nothing to disclose.

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