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Abnormal lipid/lipoprotein metabolism and high plasma testosterone levels in male but not female aromatase-knockout mice

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ABSTRACT

Sex steroid hormones, such as estrogen and testosterone, are believed to play important roles in lipid metabolism. To elucidate the effects of estrogen depletion on lipid metabolism in male and female mice, we used aromatase-knockout (ArKO) mice, in which *Cyp19* gene disruption prevented estrogen synthesis *in vivo*. These mice were divided into the following 4 groups: male and female ArKO mice and male and female wild-type (WT) mice. These mice were fed a normal-fat diet (13.6% fat) *ad libitum*. At 159 days after birth, the mice were tested for liver and plasma lipid content and hepatic hormone receptor- and lipid/lipoprotein metabolism-related gene expression. Interestingly, we found that hepatic steatosis was accompanied by markedly elevated plasma testosterone levels in male ArKO mice but not in female ArKO mice. Plasma lipoprotein profiles exhibited concurrent decreases in LDL- and small dense LDL-triglyceride (TG) levels in male ArKO mice. Moreover, male mice, but not female mice, exhibited marked elevations in androgen receptor (*AR*), sterol regulatory element-binding protein 1 (*SREBP1*), and *CD36* expression. These results strongly suggest that *Cyp19* gene disruption, which induces a sexually dimorphic response and high plasma testosterone levels in male mice, also induces hepatic steatosis.
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1. Introduction

The sex steroid hormone estrogen plays an important role in male and female reproductive function [1]. Estrogen deficiency is known to cause impaired reproductive function and is associated with the risk of developing diseases such as breast cancer, endometriosis, osteoporosis, and cardiovascular disorders in females [2].

Aromatase cytochrome P450 is a rate-limiting enzyme in estrogen biosynthesis and is encoded by the *Cyp19* gene [2,3]. Aromatase catalyzes conversion of the C19 androgen steroid substrate to a phenolic A-ring characteristic of C18 estrogens [4] in the ovaries, testes, brain, liver, muscles, and other tissues [2,3,5]. In men, *Cyp19* gene mutations decrease bone mass and cause lipid metabolism abnormalities [2,6–8]. In women, aromatase deficiency leads to pseudohermaphroditism and virilization at puberty [2,7,8].

Hepatic triglyceride (TG) accumulation causes fatty liver, metabolic abnormalities and multiple diseases [9]. One of these diseases, nonalcoholic fatty liver disease (NAFLD), varies from steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis [10]. Beyond steatosis, NASH presents as intralobular inflammation and hepatocellular ballooning, which is often accompanied by

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progressive fibrosis [11]. NAFLD has recently been reported to occur in 9–30% of Japanese adults, and NASH has been estimated to affect 1–3% of Japanese adults, although both syndromes vary in prevalence and severity depending on age and sex [12]. In Japan, NAFLD occurs in approximately 27% of men over the age of 30 years [12,13], and 23% of women develop NAFLD by the age of 30 years, but this number gradually increases in individuals over 60 years of age [12]. Moreover, NASH severity in postmenopausal women (older than approximately 50 years of age) is greater than that in men [12]. Therefore, estrogen undoubtedly also plays an important role in regulating lipid metabolism [14]. Furthermore, low serum testosterone levels have been independently associated with NAFLD in Korean men [15]. In contrast, postmenopausal American women with high serum testosterone levels have been reported to be at greater risk of developing fatty liver [16].

Aromatase-knockout (ArKO) mice, which lack the ability to synthesize estrogens *in vivo,* were previously generated through gene targeting [17–19]. Female ArKO mice undergo anovulation and become infertile [18,20]. Male ArKO mice develop glucose intolerance, insulin resistance, and obesity [14,21]. Furthermore, Jones et al. [22,23] have reported that female and male ArKO mice accumulate much more intra-abdominal adipose tissue than do wild-type (WT) mice and exhibit elevated serum leptin and cholesterol (Cho) levels compared with the levels in WT mice. Female ArKO mice exhibit elevated serum Cho levels, whereas male ArKO mice exhibit abnormal increases in hepatic Cho content [24].

In the present study, we investigated the sexually dimorphic response to *Cyp19* disruption and the effects of estrogen deficiency on serum and hepatic lipid metabolism in male and female ArKO mice fed a normal-fat diet.

2. Materials and methods

2.1. Animals, genotyping, and experimental protocols

Animal experiments were carried out in accordance with the animal care and use protocol approved by the Institutional Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology (TMIG) (Permit Number: 15005) and in accordance with the Guidelines for the Care and Use of Laboratory Animals of the TMIG.

ArKO mice were generated by using a previously described gene targeting technique [17–19]. Because male ArKO mice are known to exhibit impaired mounting behavior [19], we bred ArKO mice by using sperm from male ArKO mice and ova from female *Cyp19* heterozygote mice and performed *in vitro* fertilization and embryo transfer, as described previously [25]. The resulting ArKO offspring were identified by polymerase chain reaction (PCR) using genomic DNA isolated from tail tips [19]. Briefly, mutant *Cyp19* PCR products were amplified by PCR using genomic DNA and forward (5′-GCAGCCCCTGACACCATGTC-3′) and reverse (5′-AACTTTCATCATCACCATGGCGATGTACTT-3′) primers and were analyzed by agarose gel electrophoresis. The mutant *Cyp19* gene showed a 6.5 kbp band, and the WT allele showed a 5.0 kbp band. C57BL/6JJcl mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). At 42 days of age, these mice were divided into the following 4 groups: male ArKO ($N = 5$) mice, male WT ($N = 5$) mice, female ArKO ($N = 4$) mice, and female WT ($N = 5$) mice. All mice were fed a normal-fat diet (CRF-1) (Oriental Yeast Ltd., Tokyo, Japan) [26] containing 13.6% of calories from fat, 24.5% of calories from protein, and 61.9% of calories from carbohydrate *ad libitum* until the experiments ended. Throughout the experiments, the animals were maintained at 22 ± 1 °C and 55± 5% relative humidity under a 12-h light/dark cycle in a controlled environment.

2.2. Plasma and tissue collection

All mice were fasted for 3 h and anesthetized with sodium pentobarbital (Sumitomo Dainippon Pharma Co., Ltd., Tokyo, Japan) at 159 days after birth (23 weeks). Blood was collected from the inferior vena cava, anticoagulated with ethylenediaminetetraacetic acid (EDTA) and subsequently centrifuged at 880 × g for 15 min at 4 °C. The supernatant was divided to separate the plasma. After perfusion with ice-cold phosphate buffered saline through the left ventricle to wash out blood, the liver, kidneys, heart, epididymal adipose tissue, ovaries, uterus, and periovular adipose tissue were removed from each mouse. The livers were immersed in RNAlater® (Life Technologies Corp., Carlsbad, CA, USA) for RNA extraction and in Tissue-Tek® O.C.T. Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) for preparation of frozen sections. The livers and other tissues were frozen in liquid nitrogen for biochemical analysis or fixed with 10% neutral buffered formalin (Mildform® 10 N, Wako Pure Chemical, Osaka, Japan) for histological analysis. The formalin-fixed samples were stored at 4 °C, and the other samples were stored at −80 °C until use.

2.3. Biochemical blood analysis

Plasma total protein (TP), blood urea nitrogen (BUN), creatinine (CRE), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were assessed via biochemical analysis (Oriental Yeast Co., Ltd., Tokyo, Japan).

2.4. Plasma testosterone level measurement

Plasma testosterone levels were measured using a Testosterone EIA Kit (product no. 582701, Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions [27,28]. The cross-reactivities of the antiserum in this kit with 5α-dihydrotestosterone, 5β-dihydrotestosterone, and the other steroids were 27, 19%, and <5% of testosterone, respectively.

2.5. Liver lipid measurement

Total hepatic lipids were extracted by using the modified Bligh and Dyer's method [29–31]. Briefly, the livers were homogenized with 2 vol of ultrapure water using a Potter-Elvehjem Teflon homogenizer. The homogenates were added to chloroform-methanol (2:1; v/v, Wako Pure Chemical Industries, Osaka, Japan) and mixed at 37 °C for 1 h. After incubation, the samples were centrifuged at 21,000 × g for 10 min at 4 °C. The lower organic phase was then collected and dried at 55 °C under nitrogen gas and dissolved in 2-propanol (Sigma-Aldrich, St. Louis, MO, USA). Total cholesterol (T-cho), TG, phospholipid (PL), and non-esterified fatty acid (NEFA) levels in the total lipid extracts from the livers were measured by using enzymatic assay kits (Wako Pure Chemical Industries, Osaka, Japan).

2.6. Histological examination of the liver, testes, ovaries, and uterus

To evaluate histological changes, we subjected fixed tissue sections to hematoxylin-eosin (HE) staining. For lipid staining, we prepared frozen liver sections on glass slides (New Silane II, Muto Pure Chemicals Col, Ltd., Tokyo, Japan) before staining the sections with oil red O [32] and counterstaining them with hematoxylin.

2.7. High-performance liquid chromatography (HPLC) analysis of plasma lipoproteins

Plasma lipoprotein levels were determined using an HPLC

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