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Treatment with estrogen protects against ovariectomy-induced hepatic steatosis by increasing AQP7 expression

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Abstract. Recent evidence has suggested that the marked decrease in ovarian secretion of estrogens in postmenopausal women may be associated with the development of non-alcoholic fatty liver disease. The present study aimed to elucidate the mechanisms by which low levels of estrogen induce fatty liver disease using an ovariectomized (OVX) mouse model and an *in vitro* cell model. A total of 24 female C57/BL6 mice were divided into four groups: Sham operation, sham operation plus subcutaneous implantation of tamoxifen (TAM), bilateral OVX, and OVX plus subcutaneous implantation of 17β-estradiol (E2). Marked hepatic steatosis and increased expression of lipogenic genes (acetyl-CoA carboxylase, fatty acid synthase and glycerol-3-phosphate acyltransferase) was observed in the estrogen-depleted mice (TAM and OVX groups), as compared with in the sham operation group. Treatment with E2 significantly improved hepatic steatosis by decreasing the expression of the aforementioned lipogenic genes. Furthermore, hepatic aquaporin 7 (AQP7) expression was decreased in the estrogen-depleted mice, but was increased in the OVX + E2 treatment group, as compared with in the sham operation group. These results suggested an association between AQP7 and low estrogen-induced hepatic steatosis. Subsequently, the functions of AQP7 in hepatic steatosis were investigated using an oleic acid-induced HepG2 cell model of steatosis. Treatment with E2 alleviated lipid accumulation and decreased the expression of lipogenic genes *in vitro*; however, such effects were attenuated following transfection with AQP7 small interfering RNA. The present study suggested a mechanism by which low levels of estrogen induce fatty liver disease, and may provide useful information regarding the prevention and treatment of fatty liver disease in postmenopausal women.

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Key words: aquaporin 7, estrogen, hepatic steatosis, lipogenesis

Introduction

Menopause is a natural process that occurs in every woman's life cycle. Due to the increasing average lifespan of humans, ~40% of women's lives occur after menopause. The marked decrease in ovarian secretion of estrogens that occurs around menopause has been hypothesized as a main cause of numerous diseases, including osteoporosis and stroke (1,2). In addition, the association between menopause and pathophysiological alterations in the liver, which is the primary target organ of estrogen, has recently attracted more attention. Non-alcoholic fatty liver disease (NAFLD), which is characterized by excessive hepatic lipid accumulation due to causes other than significant alcohol consumption, is considered a risk factor for type 2 diabetes and cardiovascular disease (3). Excessive hepatic fat accumulation can be caused by increased hepatic fat synthesis and delivery, and by reduced fat oxidation and exportation. It has previously been reported that the prevalence of NAFLD is lower in premenopausal women than in men, whereas the prevalence markedly increases in postmenopausal women, consequently exceeding that in men (4). Basic and clinical studies have suggested that estrogens protect from the development of NAFLD (5,6); however, little is currently known regarding the mechanisms by which low levels of estrogen contribute to fatty liver disease. Clarifying the underlying mechanisms may provide useful information regarding the prevention and treatment of fatty liver disease in postmenopausal women.

Dysregulated hepatic lipid metabolism is considered a prerequisite for the development of NAFLD. Triglyceride (TG), which is the product of a condensation reaction between glycerol and free fatty acids, is the main form of hepatic lipid. An imbalance between the synthesis and secretion of TG results in its accumulation in liver cells, which is the main feature of fatty liver disease (7). Previous studies have reported an association between aquaporin 7 (AQP7), a water-glycerol transporter, and adult-onset obesity. In adipocytes, AQP7 deficiency increased glycerol kinase activity, enhancing TG synthesis and ultimately leading to obesity (8,9). Given the close relationship between AQP7 and lipid metabolism, the present study hypothesized that AQP7 may serve a role in other tissues with active lipid metabolism, such as the liver. AQP2, another member of the AQP family, has been identified as a target of estrogen, which mediates estrogen-enhanced migration and invasion of Ishikawa cells (10). Therefore, the present

study aimed to determine whether AQP7 may act as a target gene of estrogen and serve a role in low level estrogen-induced fatty liver disease.

The present study investigated the role of AQP7 in low estrogen-induced hepatic steatosis using an ovariectomized (OVX) mouse model. To gain further insights into the underlying mechanism of action, the effects of AQP7-specific small interfering (si)RNA and estrogen were determined in an oleic acid (OA)-induced cell model of steatosis.

Materials and methods

Experimental design. All experiments were approved by the ethics committee of the Zhujiang Hospital of Southern Medical University, (Guangzhou, China). C57/BL6 female mice (age, 6 weeks) were purchased from the Shanghai Experimental Animal Center (Shanghai, China), and were maintained under standard conditions in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (11). Mice were randomly divided into four groups (n=6/group): Group 1, which underwent a sham operation; group 2, which underwent a sham operation and were administered a subcutaneous implantation of tamoxifen [TAM; Innovative Research of America (Sarasota, FL, USA)] sustained release tablet; group 3, which underwent bilateral ovariectomy (OVX); and group 4, which underwent OVX and were administered a subcutaneous implantation of 17β-estradiol sustained release tablet (E2; 0.25 mg/pellet; 60-day release; Innovative Research of America, Sarasota, FL, USA) at the time of OVX. All mice were anesthetized anaesthetized with sodium pentobarbitol (0.04 mg/g; Propbs Biotechnology, Beijing, China.) In the OVX group, after an abdominal incision was performed, ligation was placed around the oviduct and blood vessels, the ovaries were removed through an incision, and the incision was closed with suture. For the sham operation, group, abdominal incision was performed followed by closure with suture. All mice were sacrificed by cervical dislocation 8 weeks post-treatment. Liver specimens were subsequently excised, and were stored in liquid nitrogen or fixed in 4% (w/v) paraformaldehyde.

Histological analysis of tissues. Liver samples were fixed with 4% (w/v) paraformaldehyde overnight. Sections (5-μm) were prepared from the paraffin-embedded tissues and were analyzed by hematoxylin-eosin (H&E) staining. For Oil Red O (ORO) staining, 8-μm sections were prepared from the frozen tissues, and were stained with ORO (Sigma-Aldrich, St. Louis, MO, USA) and lightly counterstained with hematoxylin, as described previously (12) and visualized using a light microscope (ECLIPSE Ti-S; Nikon Corporation, Tokyo, Japan).

Cell culture and OA-induced steatosis. HepG2 cells were purchased from the Cell Bank of the Shanghai Branch of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) and 100 U/ml penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The HepG2 cells were seeded into 6-well plates and were divided into four groups: siRNA negative control (siNC)-transfected group, siAQP7-transfected group, siNC + estradiol (E2)-treated group, and siAQP7 + E2-treated group.

HepG2 cells were transfected with the indicated siRNA oligonucleotides (Shanghai GenePharma Co., Ltd., Shanghai, China; siAQP7, TAGCCATGAACTCTGGATTGT) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, siRNA (50 pmol) and 5 μl Lipofectamine® 2000 were incubated separately with Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) for 5 min, and were then mixed together for 20 min at room temperature. Subsequently, the mixture was applied to the cells plated in 2 ml medium. A total of 48 h post-transfection, the HepG2 cells were treated with 1 mM OA solution (Sigma-Aldrich), and E2-bovine serum albumin solution (1 μM; Sigma-Aldrich) was added to the E2-treated group. After 24 h, the medium was removed and the cells were fixed with 4% paraformaldehyde at room temperature for 10 min; the cells were then stained with ORO solution (Sigma-Aldrich) and lightly counterstained with hematoxylin. After the cells were dried and mounted with glycerin, they were examined under a light microscope (ECLIPSE Ti-S; Nikon Corporation). Red oil droplets in the cells were considered to indicate OA-induced steatosis.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the liver tissues or from HepG2 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following homogenization with a tissue grinder in TRIzol®, according to the manufacturer's protocol. DNase I-treated (Sigma-Aldrich) RNA was reverse transcribed using the Superscript III enzyme (Invitrogen; Thermo Fisher Scientific, Inc.). DNase I-treated RNA was reverse transcribed using the Superscript III enzyme (Invitrogen; Thermo Fisher Scientific, Inc.) by incubation at 37˚C for 60 min, 85˚C for 5 min and 4˚C for 5 min. qPCR was performed with 5 μl SYBRGreen Mix (Thermo Fisher Scientific, Inc.), 0.5 μl forward primer, 0.5 μl reverse primer, 2 μl cDNA template and 9.5 μl ddH_2O, on an ABI 7300 real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers were purchased from Generay (Shanghai, China) Thermal cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 45 sec. Primer sequences are listed in Table I. Gene expression was determined using the $\Delta\Delta$Cq method (13). All data represent the average of three replicates.

Western blot analysis. Protein was extracted from ~0.2 g of tissue sample by homogenization with a tissue grinder in radioimmunoprecipitation assay cell lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and the protein concentration in the lysates was quantified using an enhanced bicinchoninic acid protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Equal amounts of protein (50-80 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electroblotted onto nitrocellulose membranes (EMD Millipore, Bedford, MA, USA). The blots were blocked with 5% nonfat dry milk for 1 h and were then incubated with primary antibodies [AQP7 (rabbit polyclonal antibody, 1:500 cat no. ab32826), fatty

Table I. Primer sequences for quantitative polymerase chain reaction.

Primer	Primer sequence	Size (bp)
AQP7 (*Mus musculus*) (NM_007473.4)	F: 5'-TGTCTCTTCGCCATCACC-3' R: 5'-CCACCACCAGTTGTTTCC-3'	213
ACC (*Mus musculus*) (NM_133904.2)	F: 5'-TGCTCCAGGCTAAGCGATTC-3' R: 5'-ATGCCCACCTCGTTACAACC-3'	208
FAS (*Mus musculus*) (NM_007988)	F: 5'-GGGTGGATGCAACTTTAATG-3' R: 5'-AAAGCACCAGTTCACAGATG-3'	134
GPAT (*Mus musculus*) (NM_008149.3)	F: 5'-GTTCATCCAGTATGGCATTC-3' R: 5'-TTCATCTTCCTCGTCACTTC-3'	130
GAPDH (*Mus musculus*) (NM_008084.2)	F: 5'-ATCACTGCCACCCAGAAG-3' R: 5'-TCCACGACGGACACATTG-3'	191
AQP7 (*Homo sapiens*) (NM_001170.1)	F: 5'-ACGGACCAGGAGAACAAC-3' R: 5'-CCCAACCAGCAATGAAGG-3'	160
ACC (*Homo sapiens*) (NM_198834.2)	F: 5'-GCAGGTATCCCAACTCTTC-3' R: 5'-GTAGCCCATCATCCACATC-3'	139
FAS (*Homo sapiens*) (NM_004104.4)	F: 5'-TGCCAAGAAGGGAAGGAG-3' R: 5'-TGGTGTTGCTGGTGAGTG-3'	238
GPAT(*Homo sapiens*) (NM_001244949.1)	F: 5'-GCTGCTCACTTTCATTCTC-3' R: 5'-ACATCTTTCCGTCCATCTG-3'	159
GAPDH (*Homo sapiens*) (NM_001256799.1)	F: 5'-CACCCACTCCTCCACCTTTG-3' R: 5'-CCACCACCCTGTTGCTGTAG-3'	110

AQP7, aquaporin 7; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse; bp, base pairs.

acid synthase (FAS; rabbit monoclonal antibody; 1:1,000; cat no. ab128870), glycerol-3-phosphate acyltransferase (GPAT; rabbit polyclonal antibody, 1:800; Sigma-Aldrich; cat no. PRS4613), acetyl-CoA carboxylase (ACC; rabbit polyclonal antibody; 1:800; cat no. 3662), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; rabbit monoclonal antibody; 1:1500; cat no. 5174) both Cell Signaling Technology, Inc. Danvers, MA, USA] overnight at 4˚C, followed by appropriate polycolonal goat anti-rabbit secondary antibody; Beyotime Institute of Biotechnology, Haimen, China (1:1,000; cat no. A0208) for 1 h at room temperature. Detection of specific proteins was performed by enhanced chemiluminescence (EMD Millipore). Band density was measured using ImageJ software (version 1.49; National Institutes of Health, Bethesda, MD, USA) and expression levels were normalized to GAPDH. Antibodies against AQP7, FAS and GPAT were purchased from Abcam (Cambridge, MA, USA), and antibodies against ACC and GAPDH were purchased from Cell Signaling Technology, Inc.

Determination of TG contents. Cell lysates were prepared as aforementioned, and TG contents in the cell lysates were determined using a colorimetric assay (TG assay kit; Nanjing Jiancheng Bioengineering Institute). Results were expressed as mol of TG/g of cellular protein, as described previously (14).

Statistical analysis. Data are presented as the mean ± standard deviation. Data was analyzed using GraphPad Software (version 6.0; GraphPad Software, Inc. La Jolla, CA, USA) Differences between the groups were determined by one-way analysis of variance, followed by Sidak's test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Estrogen protects mice from OVX-induced hepatic steatosis. OVX is often used as an experimental animal model to simulate menopause (15,16), and TAM is an antagonist to estrogen (17). In order to study the effects of estrogen on hepatic steatosis, 24 mice were randomly divided into four groups (n=6/group): Sham operation group, sham operation + TAM group, OVX group, or OVX + E2 group. After 8 weeks, liver specimens were collected. Histological examination of the H&E-stained liver sections revealed that TAM or OVX led to marked fat accumulation in the liver specimens, as compared with in the sham operation group (Fig. 1A). Moderate to marked macrovesicular steatosis without pattern was present throughout the hepatic lobule, whereas mice in the sham operation group displayed minimal evidence of hepatic fat accumulation. Furthermore, treatment with E2 alleviated hepatic steatosis in the mice following OVX. ORO staining further confirmed that lipid accumulation was excessive in the livers of the sham operation + TAM and OVX groups (Fig. 1B), whereas it was normal in the sham operation and OVX + E2 groups. These

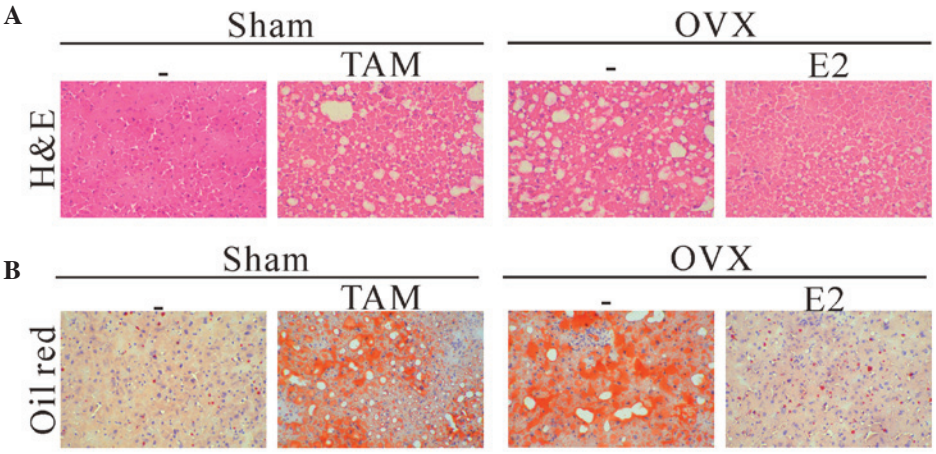

Figure 1. Treatment with estrogen reduced ovariectomy (OVX)-induced fatty liver disease. (A) Hematoxylin-eosin (H&E)-stained liver sections. (B) Oil red O staining of liver lipid droplets. Magnification, x200. TAM, tamoxifen; E2, 17β-estradiol.

data indicate that estrogen may exert inhibitory effects on hepatic steatosis.

Estrogen suppresses the expression of lipogenic genes. To gain further insight into the mechanisms underlying the protective effects of estrogen against liver steatosis, the present study analyzed the hepatic expression levels of genes associated with lipogenesis and fatty acid oxidation. As shown in Fig. 2, the mRNA and protein expression levels of lipogenic genes: ACC, FAS and GPAT, were significantly increased in the livers of the sham + TAM and OVX groups, as compared with in the sham operation group. Treatment with E2 notably decreased the expression levels of these genes. These data suggest that estrogen may reduce liver lipid accumulation via inhibiting the expression of lipogenic genes.

Estrogen increases the expression of AQP7. To investigate the involvement of AQP7 in OVX-induced hepatic steatosis, the present study detected the hepatic mRNA and protein expression levels of AQP7 in the four groups. As shown in Fig. 3, TAM treatment and OVX significantly decreased the mRNA and protein expression levels of AQP7, as compared with in the sham operation group. Treatment with E2 at the time of OVX markedly increased the expression levels of AQP7. These data provide evidence of an association between AQP7 and OVX-induced hepatic steatosis.

Estrogen improves OA-induced steatosis in HepG2 cells. In order to determine the function of estrogen and AQP7 on hepatic steatosis *in vitro*, an OA-induced steatosis model was established in HepG2 cells, as described previously (18). Cells were divided into four groups: Cells transfected with siNC, cells transfected with siAQP7, cells transfected with siNC and treated with E2, and cells transfected with siAQP7 and treated with E2. In line with the *in vivo* findings, treatment with E2 *in vitro* increased the mRNA and protein expression levels of AQP7 by 69.3 and 66.6%, respectively. AQP7-specific siRNA efficiently suppressed the expression of AQP7; the efficiency was >65% (Fig. 4A and B).

Following a 24-h incubation with OA, HepG2 cells in the siNC-transfected group developed marked steatosis, which

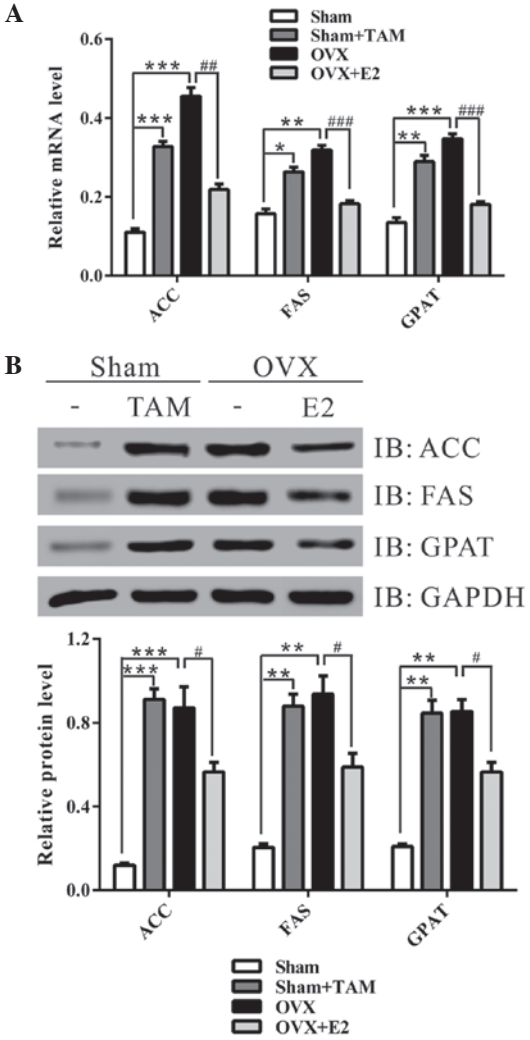

Figure 2. Treatment with estrogen at the time of ovariectomy (OVX) decreased the expression of lipid-associated enzymes. (A) Quantitative polymerase chain reaction and (B) western blot analysis of the liver specimens from the four groups. Representative western blot images are presented. Data from three independent experiments are presented as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. Sham group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. OVX group. TAM, tamoxifen; E2, 17β-estradiol; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IB, immunoblot.

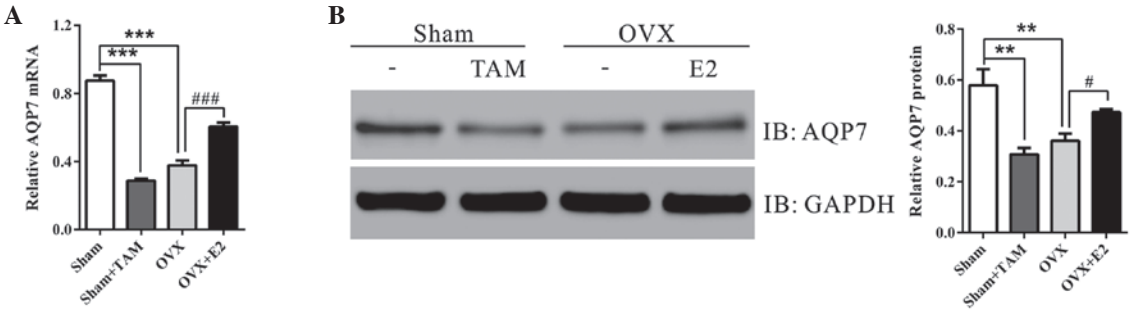

Figure 3. Estrogen increased hepatic aquaporin 7 (AQP7) expression. (A) Quantitative polymerase chain reaction analysis of AQP7 expression in the liver specimens. (B) Western blot analysis of AQP7 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression in the liver specimens. Representative western blot images are presented. Data from three independent experiments are presented as the mean ± standard deviation. **P<0.01, ***P<0.001 vs. Sham group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. ovariectomy (OVX) group. TAM, tamoxifen; E2, 17β-estradiol; IB, immunoblot.

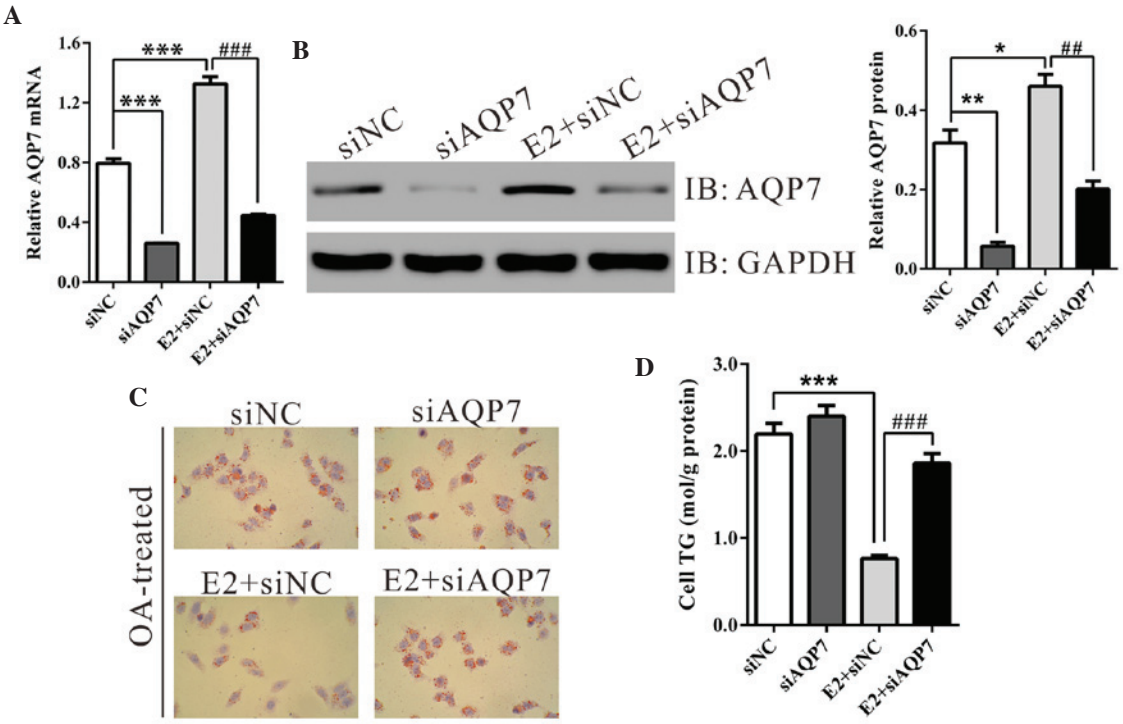

Figure 4. Estrogen improved oleic acid (OA)-induced steatosis in HepG2 cells via increasing the expression of aquaporin 7 (AQP7). AQP7 expression was determined by (A) quantitative polymerase chain reaction and (B) western blot analysis. (C) Steatosis was evaluated by Oil red O staining. (D) Triglyceride (TG) contents were measured and expressed as mol of TG/g of cellular protein. Data from three independent experiments are presented as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. negative control small interfering RNA (siNC) cells; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. estradiol (E2) + siNC cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IB, immunoblot.

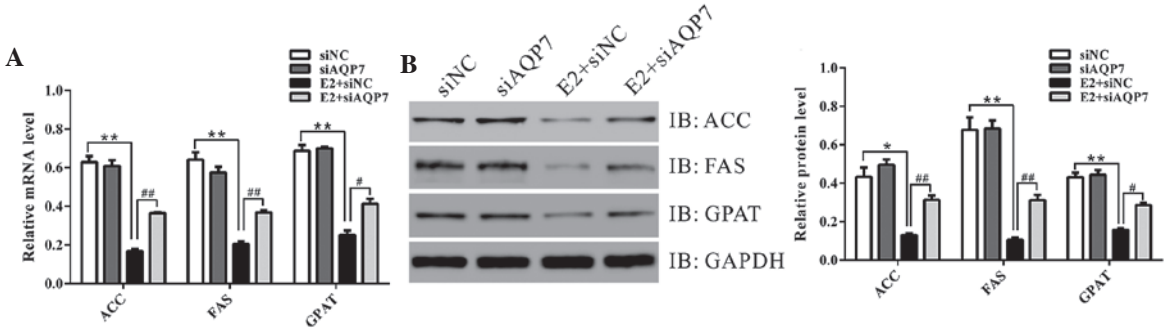

Figure 5. Expression of lipid-associated enzymes in oleic acid (OA)-induced HepG2 steatosis. (A) Quantitative polymerase chain reaction and (B) western blot analysis of lipid-associated enzymes in HepG2 cells. Data from three independent experiments are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. negative control small interfering RNA (siNC) cells; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. estradiol (E2) + siNC cells. AQP7, aquaporin 7; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IB, immunoblot.

manifested as an accumulation of lipid droplets in the cytoplasm. Treatment with E2 significantly decreased the number of lipid droplets (Fig. 4C), whereas transfection of the HepG2 cells with siAQP7 attenuated the protective effects of E2 on lipid accumulation.

Consistent with these alterations, a marked reduction in TG content was observed in the E2-treated group, as compared with in the corresponding control group (siNC, 2.19±0.13; E2 + siNC, 0.76±0.04; Fig. 4D). siAQP7 transfection significantly increased TG content by 144% in cells treated with E2. These data indicate that estrogen may protect hepatocytes from steatosis via increasing the expression of AQP7.

AQP7 siRNA suppresses the expression of lipid-associated enzymes in vitro. The present study analyzed the expression levels of genes associated with lipogenesis and fatty acid oxidation *in vitro*. Treatment with E2 resulted in a marked decrease in ACC, FAS and GPAT expression (Fig. 5), which was partially inhibited by knockdown of AQP7 expression. These data suggest that estrogen may suppress lipogenesis via increasing AQP7 expression.

Discussion

Human menopause is associated with an elevated risk of NAFLD; however, the underlying molecular mechanisms remain unclear. The present study evaluated the effects of estrogen depletion on hepatic steatosis using an OVX mouse model (Figs. 1 and 2), which is often used as an experimental animal model to simulate menopause, and is associated with increased risk of bone loss (19), visceral obesity (20) and hepatic steatosis (21). Liver specimens from the OVX or TAM (estrogen antagonist)-treated mice displayed visible steatosis with increased expression of lipogenic genes (ACC, FAS and GPAT), whereas E2 treatment alleviated OVX-induced hepatic steatosis and decreased the expression levels of lipogenic genes. The present study proposed an inhibitory effect of E2 on lipogenesis and lipid accumulation, which was consistent with previous studies in human adipose tissue (22) and rat liver (23).

Estrogen regulates human physiology via signaling through intracellular hormone-specific estrogen receptors (ERs). Dimeric ERs directly bind to specific DNA sequences of target genes, known as estrogen response elements (EREs), thus mediating their expression (24). Functional EREs have been detected in AQPs, including AQP5 (25) and AQP2 (10). Previous studies have demonstrated that AQP7 modulates adipocyte glycerol permeability, thereby controlling TG accumulation and fat cell size (8,9). The present study hypothesized that AQP7 may act as a target gene of estrogen and serve a role in OVX-induced hepatic steatosis. The results demonstrated that OVX and TAM treatment significantly decreased the hepatic expression levels of AQP7 (Fig. 3), thus suggesting the involvement of AQP7 in low estrogen-induced lipid accumulation. In order to further investigate the function of AQP7 during estrogen depletion-induced lipid accumulation, AQP7-specific siRNA and an OA-induced HepG2 cell model of steatosis was used. Treatment with E2 decreased lipid drop formation and TG content in HepG2 cells, whereas such effects were attenuated by AQP7 siRNA (Fig. 4). Analysis of lipogenic gene expression (Fig. 5) further indicated that estrogen was able to decrease lipogenesis by increasing AQP7 expression; however, the mechanisms underlying the hormone-induced regulation of AQP7 transcription require further in-depth investigation.

In conclusion, the present study indicated that estrogen exposure alleviated hepatic steatosis by regulating the expression of its target, AQP7, in an OVX mouse model and a cellular model. In addition to its function in glycerol transport, AQP7 serves an important role in lipogenesis. The present study provided potential targets for the prevention and treatment of fatty liver disease in postmenopausal women.

Acknowledgements

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