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Estrogen upregulates hepatic apolipoprotein M expression via the estrogen receptor

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ABSTRACT

Apolipoprotein M (apoM) is present predominantly in high-density lipoprotein (HDL) in human plasma, thus possibly involved in the regulation of HDL metabolism and the process of atherosclerosis. Although estrogen replacement therapy increases serum levels of apoAI and HDL, it does not seem to reduce the cardiovascular risk in postmenopausal women. Therefore, we investigated the effects of estrogen on apoM expression *in vitro* and *in vivo*. HepG2 cells were incubated with different concentrations of estrogen with or without the estrogen receptor antagonist, fulvestrant, and apoM expression in the cells was determined. Hepatic apoM expression and serum levels of apoM were also determined in normal and in ovariectomized rats treated with either placebo or estradiol benzoate, using sham operated rats as controls. Estrogen significantly increased mRNA levels of apoM and apoAI in HepG2 cell cultures in a dose- and time-dependent manner; the upregulation of both apolipoproteins was fully abolished by addition of estrogen receptor antagonist. In normal rats, estrogen treatment led to an increase in plasma lipid levels including HDL cholesterol, a marked upregulation of apoM mRNA and a significant increase in serum levels of apoM. The same pattern of regulation was found in ovariectomized rats treated with estrogen. Thus, estrogen upregulates apoM expression both *in vivo* and *in vitro* by mechanism(s) involving the estrogen receptor.

1. Introduction

Estrogens are a group of steroid compounds, named for their importance in the estrous cycle, and function as the primary female sex hormone. It is well established that estrogens are actively involved in the lipid metabolism both in physiological and pharmacological contexts. For example, during supplementation treatment of postmenopausal women with conjugated equine estrogen, plasma concentrations of total cholesterol (TC), low-density lipoproteins (LDL) and apolipoprotein B (apoB) tend to decrease, while the levels of triglycerides (TG), high-density lipoproteins (HDL) and apoA-I increase significantly [1–3]. In general, these effects of estrogen are considered beneficial from the atherogenic point of view. However, randomised, controlled trials of estrogen supplementation in postmenopausal women have failed to demonstrate a benefit of estrogens in the primary or secondary prevention of cardiovascular diseases (CVD), although estrogens might be effective if targeted at younger perimenopausal women [5].

Apolipoprotein M (apoM), one of the most recently discovered serum apolipoproteins, is mainly associated with HDL, with only a small proportion located in LDL and very low density lipoprotein (VLDL) particles [6]. In humans and rats, apoM is mainly expressed in hepatocytes and in kidney proximal tubule epithelial cells [7,8]. About 5% of total HDL contains apoM in human plasma, and apoM is associated with a heterogeneous subpopulation of HDL particles [9]. Although apoM is not present in all HDL particles, recent data have demonstrated a positive correlation between serum apoM levels and HDL cholesterol concentrations in man [10]. Wolfrum and his colleagues, using apoM-deficient mice, demonstrated that apoM is important for preβ-HDL formation and cholesterol efflux from macrophages; thus, apoM-deficient HDL was markedly less efficient in facilitating cholesterol efflux from macrophages *in vitro* than normal HDL [11]. Moreover, over-expression of apoM in LDL-receptor knock-out mice protected against atherosclerosis when fed a high cholesterol diet [11], suggesting that apoM may play an important role in HDL metabolism and protect against atherosclerosis.

Considering the discrepancy between the effects of estrogen on HDL concentrations and atherogenicity in postmenopausal women,

Abbreviations: CAD, coronary artery disease; HDL, high density lipoprotein; OVX, ovariectomy; EB, estradiol benzoate; TG, total cholesterol; TC, total triglycerides; LDL, low-density lipoprotein; VLDL, very low density lipoprotein; FBS, fetal bovine serum; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; ECL, electrogenerated chemiluminescence; SE, standard error; PAF, platelet activating factor; TGF, transforming growth factor; EGF, epidermal growth factor; HGF, hepatic growth factor; TNF, tumor necrosis factor; IL, interleukin; ER, estrogen receptor; ERE, ER-response element

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we performed a series of experiments to investigate the role of estrogen in the regulation of apoM expression *in vitro* and in ovariectomized rats, with the possibility in mind that negative effects of estrogens on apoM metabolism might counteract the antiatherogenic effects of elevated HDL concentrations.

2. Materials and methods

2.1. Materials

The HepG2 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Six-well cell culture clusters and 75-cm^2 vented cell culture flasks were purchased from Nunc (Roskilde, Denmark). 17β-estrogen (estrogen) and fulvestrant from Sigma Chemical Co. Ltd. (Shanghai, China). Fetal bovine serum (FBS), DMEM, phenol red–free DMEM and charcoal-treated FBS were obtained from Invitrogen (Shanghai, China). 17β-estradiol benzoate (EB) was purchased from Shanghai GM Pharmaceutical Co. Ltd. (Shanghai, China). Total RNA purification kits were purchased from the Shenergy Biocolor BioScience and Technology Company (Shanghai, China). First strand cDNA synthesis kits were obtained from Fermantas (Vilnius, Lithuania). The LightCycler real-time RT-PCR System was from Roche Applied Science (Mannheim, Germany).

2.2. Cell cultures

HepG2 cells were cultured in DMEM supplemented with 10% FBS in the presence of 100 U/ml penicillin, 100 μg/ml streptomycin and 1% Glutamax at 37 °C under 5% CO_2 atmosphere. Cells were plated in 6-well cell culture clusters at a density of 5×10^4 cells/dish with phenol red–free DMEM containing 10% charcoal-treated FBS. Cell monolayers of approximately 50-70% confluence were grown for 24 hrs in the above media, then washed and incubated in serum-free medium with different concentrations of estrogen (0.1-10 μM) for 24 hrs, before extraction of total RNA. In the antagonism study, we used estrogen at 10 μM and increasing concentrations of fulvestrant (0.1-1 μM) in the culture medium. Estrogen and fulvestrant were dissolved in ethanol.

2.3. Animal models and estradiol benzoate administration

Adult female SD rats (body weight 180–205 g) were obtained from Shanghai Slac Laboratory Animal Co. (Shanghai, China), and housed in mesh stainless steel cages at constant room temperature (22 °C) with a 12-hrs light-dark cycle, with *ad libitum* access to rat chow and tap water. Animals were acclimated for one week before experiments. They were randomly divided into five groups: normal rats (normal group), normal rats treated with estradiol benzoate (EB group), one group which underwent the surgical procedure without OVX (sham group), ovariectomized rats treated with placebo (pure sesame oil) (OVX) and ovariectomized rats treated with estradiol benzoate (OVX + EB). The EB group (7 rats) and the OVX + EB group (5 rats) were treated with EB (125 μg/kg, *sc*), while the other groups (with 6, 5 and 5 animals in the normal, sham OVX groups, respectively) were injected with 0.1 ml vehicle (pure sesame oil, sc). All rats received treatments at the same time twice a week. After 12 hrs fasting, blood samples were obtained (about 1 ml each rat) from the tail vein under anesthesia for measurements of serum levels of triglycerides (TG), LDL-cholesterol, HDL-cholesterol, total cholesterol (TC) at months 1, 2 and 3 after operation. A specimen of liver tissue was sectioned and stored in liquid nitrogen at month 3. Body weights were registered at months 1, 2 and 3 after operations.

2.4. Total RNA extraction and real time PCR

Total RNA of HepG2 cells and from rat liver tissues was extracted using the total RNA purification kit according to the manufacturer's instructions. Primer Express software (Applied Biosystems) was used to design the human and rat apoM and/or apoAI primers and probes for the TaqMan based RT-PCR assay (see Table 1). Quantifications of apoM and apoAI mRNA levels are relative to the mRNA level of GAPDH or β-actin, and were performed on a LightCycler in a final volume of 25 μl. Optimal conditions were obtained with 2.5 μl of 10 × PCR buffer, 1.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM 4 × dNTPs, 0.25 μl of 5 U/μl common Taq DNA polymerase, 0.1 μl of 100 μM specific sense primer (s), 0.1 μl of 100 μM specific antisense primer(s), 0.1 μl of 100 μM specific probe(s) and 2 μl template cDNA. Finally 17.95 μl H_2O was added to the reaction mixture. The thermal cycling conditions for GAPDH, apoM, β-actin and apoAI included the following steps: 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min to do reverse transcription, and then the reaction mixture was preheated for 2 min at 50 °C and for 10 min at 95 °C to activate Taq polymerase. After that, a 40-cycle two-step PCR was performed consisting of 15 s at 95 °C and 1 min at 60 °C. Samples were amplified simultaneously in triplicates in one-assay run. The prospective amplicon of each gene was amplified and purified, then ligated into the pMD19-T vector, before the ligated product was transformed into the E. Coli JM109 competent cells. In brief, a serial dilution of extracted plasmid DNA was used to generate a standard curve by plotting the cycle threshold versus the log initial copy number of input plasmid DNA. Standard curves of apoM, apoAI, β-actin and GAPDH achieved a very high correlation coefficient ($r = 1.00$). The ratio between the target gene and GAPDH or β-actin was calculated as the relative gene expression.

2.5. Serum apoM protein measurements

ApoM concentrations in rat serum were determined by Western blot analysis [12]. In brief, 10 μl serum was diluted with PBS buffer (1:20) and 10 μl diluted samples were fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and then incubated with polyclonal rabbit anti-rat apoM antibody. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. Bands corresponding to apoM were visualized by an ECL + Plus Western blotting detection system (Amersham) and quantified by a scanner using the Quantity One software (Version 4.2.1, Bio-Rad Laboratories).

2.6. Biochemical analysis

Serum levels of total cholesterol (TC), triglycerides (TG), LDL-cholesterol and HDL-cholesterol were determined by routine methods.

Table 1
Primers and fluorescent probes for real-time RT-PCR.

gene	Forward primer	Reverse primer	Probe
Human apoM	5`-acaaagagaccccagagccc	5`-tccatggtgggagccg	5`-FAM-acctgggcctgtggtactttattgctgg-TAMRA
Human apoAI	5`-ctgggataacctggaaaaggagac	5`-ggaagtcgtccaggtagggct	5`-FAM-agatgagcaaggatctggaggaggtgaa-TAMRA
Human GAPDH	5`-ggaaggtgaaggtcggagtc	5`-cgttctcagccttgacggt	5`-FAM-tttggtcgtattgggcgcctg -TAMRA
Rat apoM	5`-acaaagagaccccagagccc	5`-tccatggtgggagccg	5`-FAM-acctgggcctgtggtactttattgctgg-TAMRA
Rat β-actin	5`-gccactgccgcatcctct	5`-ctggaagagagcctcgggg	5`-FAM-agctgcctgacggtcaggtcatcactatc -TAMRA