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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.

Keywords: Apolipoprotein M; HDL; Estrogen; Lipid metabolism.

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 [4].

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 [5]. In humans and rats, apoM is mainly expressed in hepatocytes and in kidney proximal tubule epithelial cells [6, 7]. About 5% of total HDL contains apoM in human plasma, and apoM is associated with a heterogeneous subpopulation of HDL particles [8]. 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 [9]. 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 [10]. Moreover, over-expression of apoM in LDL-receptor knock-out mice protected against atherosclerosis when fed a high cholesterol diet [10], 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, 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 ºC 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 ºC) 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 25mM

MgCl$_2$, 0.5 μl of 10mM 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$_2$O was

added to the reaction mixture. The thermal cycling conditions for GAPDH, apoM , β-actin and apoAI included the following steps: 25 ºC for 10 min, 48 ºC for 30 min and 95 ºC for 5 min to do reverse transcription, and then the reaction mixture was preheated for 2 min at 50 ºC and for 10 min at 95 ºC to activate Taq polymerase. After that, a 40-cycle two-step PCR was performed consisting of 15 s at 95 ºC and 1 min at 60 ºC. 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 [11]. 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.

2.7. Statistical analysis

Data are expressed as means ± SE. Statistical analyses were performed with the software Prism (version 4.0). Differences between groups were analyzed by one-way ANOVA followed by unpaired Student's *t*-test. A *P* value <0.05 was considered significant.

3. Results

3.1. Effects of estrogen on mRNA levels of apoM and apoAI in HepG2 cells

As shown in Fig 1A, 10 μM estrogen significantly upregulated mRNA levels of apoM and apoAI by 200% and 163% , respectively, compared to controls (p<0.01). The stimulatory effects of estrogen on mRNA levels of apoM and apoAI in the HepG2 cells were dose- and time-dependent and essentially similar (Fig 1A and 1B). Addition of the estrogen receptor antagonist, fulvestrant, to the cell cultures totally abolished the estrogen-induced up-regulation of apoAI and apoM (Fig 1C), which supports the concept that regulation of apoM and apoAI expression by estrogen is mediated via activation of the estrogen receptor. In the absence of estrogen, fulvestrant down-regulated apoM mRNA slightly but not significantly (data not shown).

3.2. Effects of estrogen on apoM synthesis, plasma apoM levels and plasma lipids in

vivo

As shown in Fig 2, one month´s treatment of normal rats with estrogen resulted in significantly increased serum concentrations of HDL cholesterol, but also of TG, TC, LDL-cholesterol. (The slight decrease in body weight, although statistically significant, is unlikely to have influenced these data.)

Consonant with the results in cell cultures, estrogen treatment of normal rats led to a 15-20-fold upregulation of hepatic mRNA, which was mirrored by a significant elevation of apoM levels in plasma (Fig 2C and D).

The same pattern was found in OVX rats. Again, estrogen treatment led to a substantial rise in hepatic apoM expression with a concomitant elevation of apoM concentrations in plasma (Fig 3). An interesting observation was the tendency to higher levels of hepatic apoM RNA in the sham operated rats than in OVX rats without estrogen treatment. Although not statistically significant, this finding suggests that basal estrogen levels are needed for maintenance of apoM production under normal conditions.

The effects of estrogen on the plasma lipid profile in ovariectomized rats (Fig 4) were qualitatively similar to that recorded in normal rats, with elevated levels of all plasma lipids including HDL. The differences in body weight between the three experimental groups do not harmonize with the changes in any of the plasma lipids, but suggests that esterogen levels (negligible in OVX, basal in sham, markedly elevated in OVX+EB) may influence body weight independently of their effects on plasma lipoproteins.

4. Discussion

ApoM is a human apolipoprotein predominantly present in HDL [5]. Previous studies have demonstrated that expression of apoM is regulated by certain cytokines and nuclear factors, including platelet activating factor (PAF), leptin, transforming growth factor-β (TGF-β), epidermal growth factor (EGF), hepatic growth factor (HGF), liver X receptor agonist, and hepatocyte nuclear factor-1α , either *in vivo* or *in vitro* [11-18], whereas it is not influenced by ACTH, tumor necrosis factor (TNF-α) or interleukin-1alpha (IL-1α) in cell cultures [12, 19]. The impact of apoM on cholesterol and lipoprotein metabolism has been investigated mainly in genetically modified mice. Wolfrum et al. demonstrated that lack of apoM expression in Tcflα$^{-/-}$ (hepatic nuclear factor 1α) mice or in apoM siRNA-injected mice leads to the formation of unusually large-sized HDL$_1$ particles and the disappearance of pre-β HDL in plasma, suggesting that apoM may play an important role in HDL metabolism, particularly with regard to the formation and/or catabolism of pre-β HDL [10]. The formation of pre-β HDL is thought to be important for reverse cholesterol transport. Overexpression of human apoM in mice increases HDL cholesterol concentrations, while apoM deficiency is associated with lower HDL cholesterol concentrations [20].

It is well known that estrogen is involved in the regulation of various aspects hepatic lipid and lipoprotein metabolism [21-24], including enhancement of apoAI synthesis and HDL formation. The present study demonstrates that estrogen also upregulates the expression of apoM *in vitro* in a time- and dose-dependent manner quite similar to that of apoAI, suggesting that increased synthesis of these apolipoproteins are

both important for the estrogen-induced enhancement of HDL synthesis. Tam et al. have reported that estrogen could significantly enhance apoAI and apoCII secretion in HepG2 cell cultures [25] under conditions similar to those in the present study, whereas stimulation of apoB and apoE synthesis occurred only after prolonged incubation with considerably higher esterogen concentrations [26].

Our *in vitro* studies also demonstrated that upregulation of the expressions of apoM, as well as of apoAI, are mediated via the estrogen receptor. However, the detailed mechanisms may differ. Upregulation of apoAI expression also involves non-specific ER-response elements (ERE) in the promoter region of apoAI [27] while no typical ERE have been found in the promoter region of apoM.

Our data from normal and ovariectomized rats confirm that the upregulation of apoM expression by estrogen occurs also *in vivo*. Estrogen treatment was accompanied by a significant increase of apoM concentrations in plasma, in parallel with the expected rise in plasma levels of apoAI and HDL-cholesterol. In addition, our data suggest that basal estrogen levels are necessary for the maintenance of hepatic apoM expression.

With our experimental protocol, estrogen treatment led to increased levels not only of triglycerides and HDL, but also of LDL and total cholesterol concentrations, whereas Böttner et al. reported that oral administration of estrogen decreased total cholesterol, LDL and HDL, and increased triglyceride levels in OVX rats [28]. These discrepancies may be related to the different experimental designs, in particular the different modes of estrogen administration.

5. Conclusion

The present study demonstrates that estrogen enhances the expression of apoM, an apolipoprotein with antiatherogenic potential present at low concentrations in HDL, and increases apoM levels in plasma by mechanisms involving the estrogen receptor. The pattern of regulation of apoM is similar to that of apoAI, and thus seems synchronized with the estrogen-induced increase in HDL levels. The effects of estrogen on plasma concentrations and apoM expression are similar in normal and ovariectomized rats, a finding which does not support the possibility that alterations in apoM turnover would be involved in the lack of cardioprotective effects of estrogen supplementation in postmenopausal women.

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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

Legend to figures

Fig.1. Effects of estrogen and fulvestrant on mRNA levels of apoAI and apoM in HepG2 cells.

HepG2 cells were cultured with estrogen at different concentrations for 24 hrs (Panel A) and with 10 μM estrogen for different time intervals (Panel B). Cells were also cultured with estrogen (10 μM) alone or together with fulvestrant (0.1 μM) for 24 hrs (Panel C). The mRNA levels of apoAI and apoM were determined as described in the materials and methods. Data (means ± SE) are given as % of controls (cells grown without estrogen). [**]$P<0.01$ vs. Control;[f]$P<0.05$ and[ff]$P<0.01$ vs. Estrogen.

Fig.2. Effects of estrogen treatment on body weight, lipid and apoM concentrations in serum, and hepatic apoM mRNA levels in normal rats.

Rats were treated with estradiol benzoate (EB) or vehicle (Control) for one month. Body weights (Panel A), serum lipid profile (Panel B), serum apoM concentrations (Panel C) and hepatic mRNA level of apoM (Panel D) were determined as described in Materials and methods. Data are means ± SE. [*]$P<0.05$ and [**]$P<0.05$ vs. Control.

Fig.3. Effects of estrogen treatment on serum apoM and hepatic apoM mRNA levels in ovariectomized rats.

Serum apoM concentrations (Panel A) and hepatic apoM mRNA levels (Panel B) were determined in ovariectomized rats treated with estradiol benzoate (OVX+EB) or with vehicle (OVX) for 3 months. A sham group treated with vehicle was also included (Sham). Serum apoM concentrations were determined every month and hepatic apoM mRNA level was measured at the end of the experiment. Data are means ± SE. [*]$P<0.05$ vs. OVX;[f]$P<0.05$ vs. Sham.

Fig.4. Effects of estrogen treatment on body weight and serum lipid concentrations in ovariectomized rats.

Serum triglycerides (TG), total cholesterol (TC), HDL and LDL were determined in the ovariectomized rats treated with estradiol benzoate (OVX+EB) or with vehicle (OVX) for three months. A sham group treated with vehicle was also included (Sham). Serum lipid profiles were determined after one (Panel A), two (Panel B) and three months (Panel C). Changes of body weight were shown in panel D. Data are means ± SE. * $P<0.05$, **, $P<0.01$ and $^{***}P<0.001$ vs. OVX; ^{f} $P<0.05$, $^{ff}P<0.01$ and ^{fff} $P<0.001$ vs. Sham.
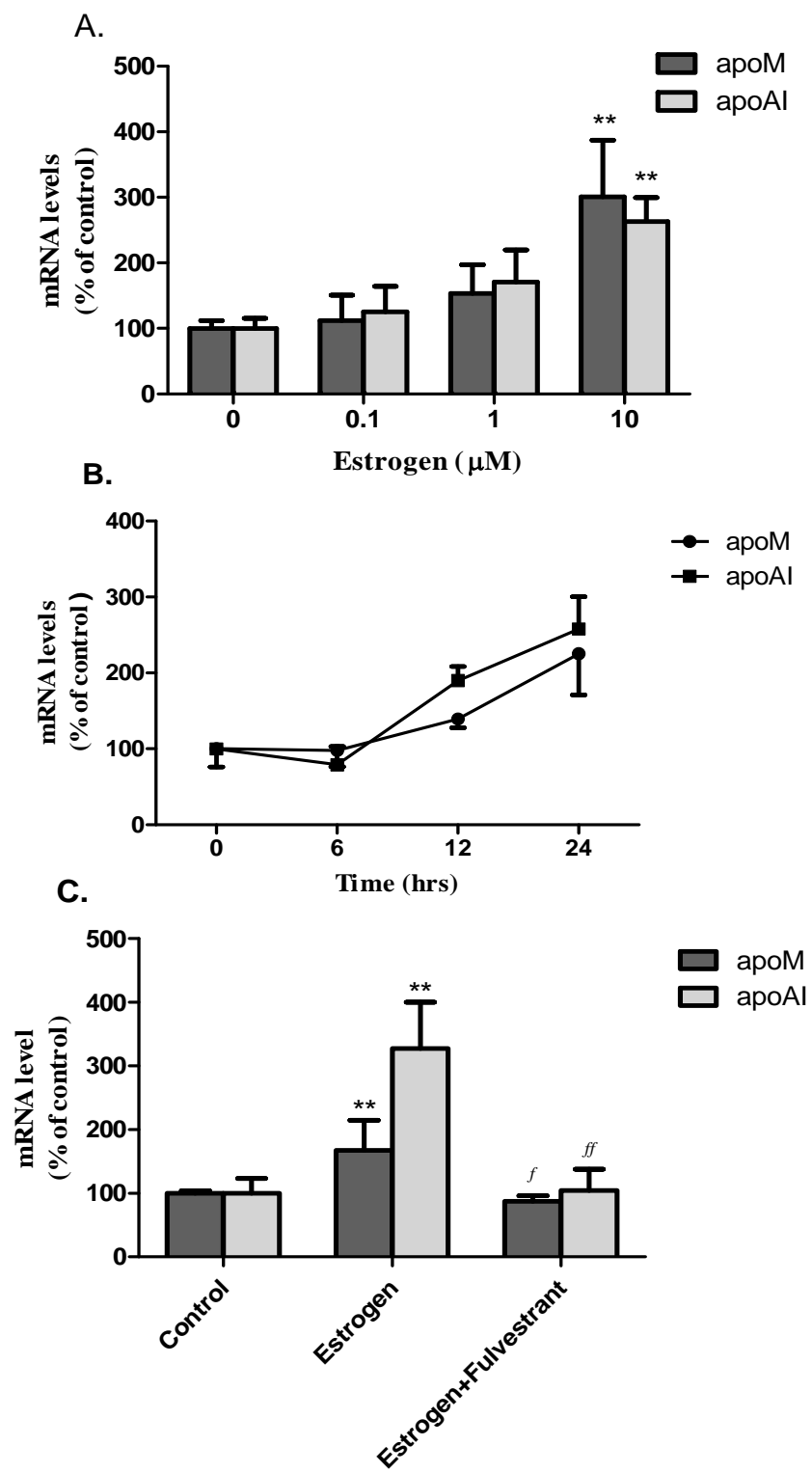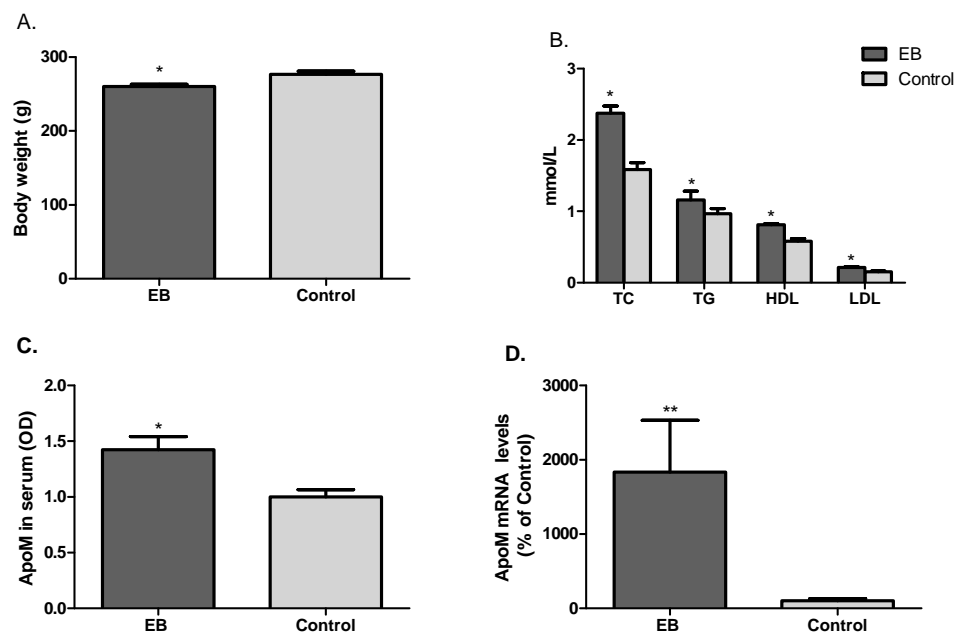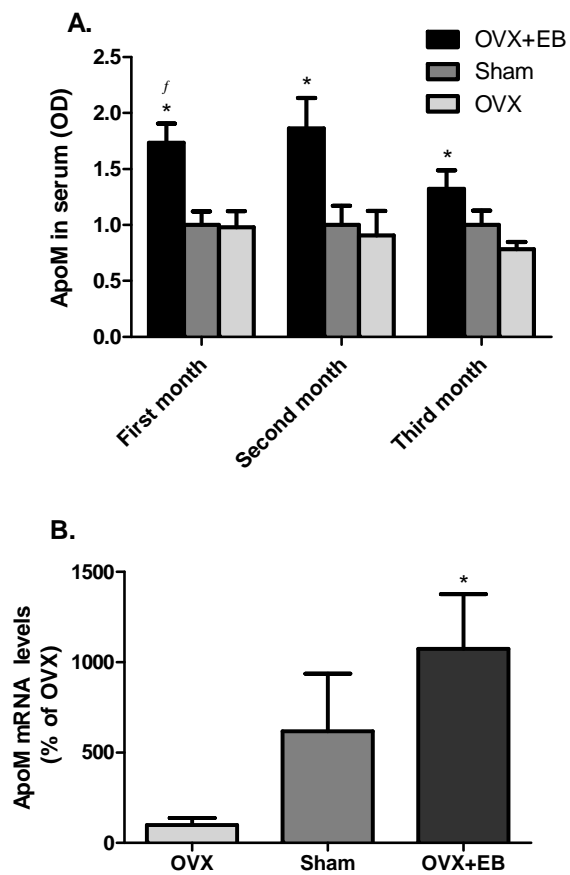
Fig 1

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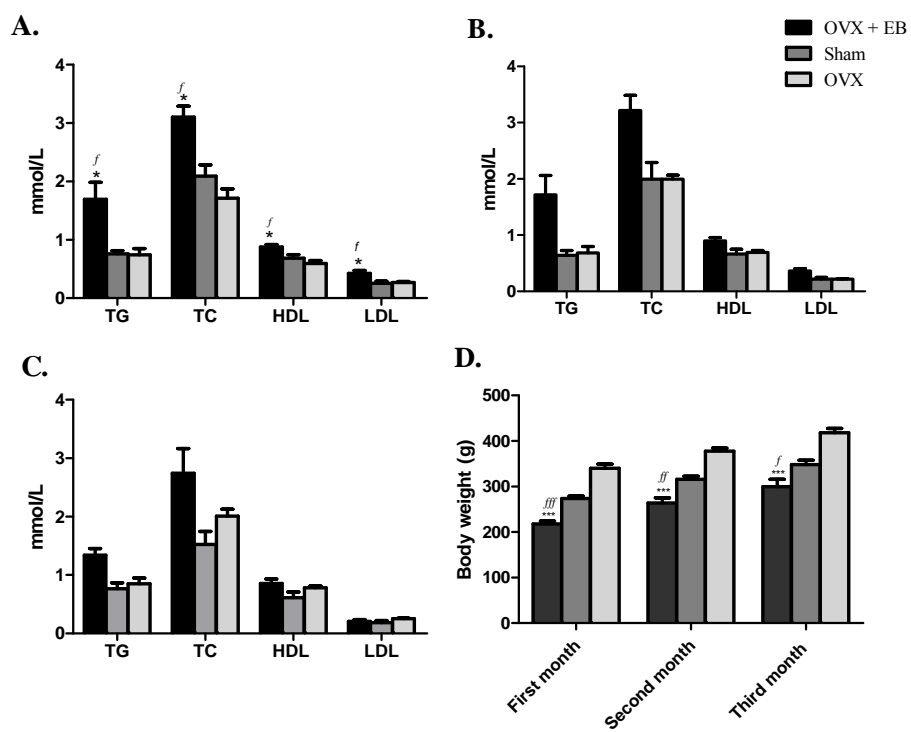
Abbreviations

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