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Unique effect of 4-hydroxyestradiol and its methylation metabolites on lipid and cholesterol profiles in ovariectomized female rats

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ABSTRACT

Animal studies have shown that endogenous estrogens such as 17β-estradiol (E_2) can modulate lipid profiles *in vivo*, and this effect is generally thought to be mediated by the estrogen receptors (ERs). The present study sought to test a hypothesis that some of the endogenous estrogen metabolites that have very weak estrogenic activity may exert some of their modulating effects on lipid metabolism in an ER-independent manner. Using ovariectomized female rats as an *in vivo* model, we found that 4-hydroxyestradiol (4-OH-E_2) has a markedly stronger effect in reducing the adipocyte size and serum cholesterol level in rats compared to E_2, despite the weaker estrogenic activity of 4-OH-E_2. Moreover, when E_2 or 4-OH-E_2 is used in combination with ICI-182,780 (an ER antagonist), some of their lipid-modulating effects are not blocked by this antiestrogen. Interestingly, two of the *O*-methylation metabolites of 4-OH-E_2, namely, 4-methoxyestradiol and 4-methoxyestrone, which have much weaker estrogenic activity, were also found to have similar lipid-modulating effects compared to 4-OH-E_2. Mechanistically, up-regulation of the expression of leptin, cytochrome P450 7A1 and LXRα genes is observed in the liver of animals treated with E_2 or 4-OH-E_2, and the up-regulation is essentially not inhibited by co-treatment with ICI-182,780. These results demonstrate that some of the endogenous E_2 metabolites are functionally important modulators of lipid metabolic profiles *in vivo*. In addition, our findings indicate that an ER-independent pathway likely mediates some of the lipid-modulating effects of endogenous estrogens and their metabolic derivatives.

1. Introduction

Estrogens play an important role in regulating lipid homeostasis in humans as well as in animal models. For instance, a number of human studies, including randomized, controlled clinical trials, have documented that estrogen therapy in postmenopausal women decreases the blood levels of total cholesterol and low-density lipoprotein (LDL) cholesterol, while increases the blood levels of high-density lipoprotein (HDL) cholesterol and triglyceride (TG) (Kuller, 2003; Mendelsohn and Karas, 1999; The Writing Group for the PEPI Trial, 1995).

Endogenous estrogens undergo extensive metabolism in animals and humans (Martucci and Fishman, 1993; Zhu and Conney, 1998), such as oxidation, inter-conversion between 17β-estradiol (E_2) and estrone (E_1), and various conjugation-deconjugation reactions. The metabolism may contribute to the diversification of the biological actions of estrogens in different target sites under different physiolo-

gical and/or pathophysiological conditions (Fishman and Martucci, 1978; Martucci and Fishman, 1993; Weisz, 1991; Zhu and Conney, 1998; Zhu and Lee, 2005).

Many of the biological actions of estrogens are mediated by the estrogen receptor (ER) α and β, although some of their effects may also be mediated by the non-genomic mechanisms or *via* the membrane ERs (Nilsson and Gustafsson, 2002). It is of note that earlier studies showed that some of the estrogen metabolites can exhibit distinct effects that are not associated with their parent hormone E_2 or their binding affinity for the ERα or ERβ. For instance, it has been reported that 2-hydroxyestradiol (2-OH-E_2) and 4-hydroxyestradiol (4-OH-E_2) can attenuate the *O*-methylation of endogenous catecholamines catalyzed by the catechol-*O*-methyltransferase, and this inhibition may modulate the neurological effects of catecholamines in the heart and central nervous system (Zhu, 2002). 2-OH-E_2 has also been suggested to have a protective effect on the cardiovascular system *via* both

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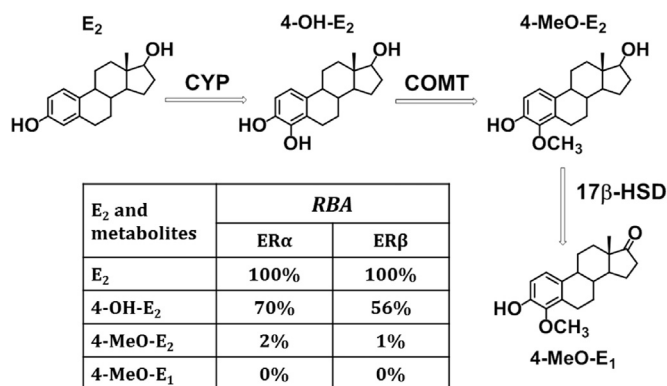*E-mail address:* BTZhu@CUHK.edu.cn (B.-T. Zhu).

Fig. 1. The chemical structures of the estrogen derivatives used in this study and the endogenous metabolic pathways (enzymes) involved in their formation. The relative binding affinity (*RBA*) of each estrogen derivative for human ERα and ERβ is shown in the inset table (taken from Zhu et al. (2006)). Abbreviations used: E_2, 17β-estradiol; 4-OH-E_2, 4-hydroxyestradiol; 4-MeO-E_2, 4-methoxyestradiol; 4-MeO-E_1, 4-methoxyestrone; CYP: cytochrome P450 enzymes; COMT, catechol-*O*-methyltranferase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

genomic and non-genomic pathways (Tofovic et al., 2001). Another earlier study reported that 4-OH-E_2 can decrease blood cholesterol levels (Liu and Bachmann, 1998).

The present study seeks to test a hypothesis that some of the endogenous estrogen metabolites which have weak or no estrogenic activity might still have a very strong effect in modulating lipid profiles *in vivo*. We used the ovariectomized (OVX) female Sprague-Dawley rats as an *in vivo* model to probe the lipid-modulating effect of 4-hydroxyestradiol (4-OH-E_2), which is an important endogenous E_2 metabolite formed by cytochrome P450 enzymes (structures and metabolic scheme are shown in Fig. 1) (Zhu and Conney, 1998). In addition, we have also tested for comparison the effect of 4-methoxyestradiol (4-MeO-E_2) and 4-methoxyestrone (4-MeO-E_1), two of the major endogenous metabolites of 4-OH-E_2 (Fig. 1) that have little or no binding affinity for human ERα and ERβ (Zhu and Conney, 1998). To help probe the involvement of ERs in mediating the lipid-modulating effect of E_2 and 4-OH-E_2, ICI-182,780, a pure antagonist for the ERs, was used as a tool drug for this purpose. We found that 4-OH-E_2, 4-MeO-E_2 and 4-MeO-E_1 have a strong effect in altering some of the lipid profiles in the OVX rats, and some of these effects appear to be independent of the ERs.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (E_2), 4-hydroxyestradiol (4-OH-E_2), 4-methoxyestradiol (4-MeO-E_2) and 4-methoxyestrone (4-MeO-E_1) were obtained from Steraloids (Newport, RI, USA), and ICI-182,780 was obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The organic solvents used in this study were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). TRIzol was obtained from Invitrogen (Carlsbad, CA, USA). MuLV reverse transcriptase and the SYBR Green PCR Master Mix were obtained from Applied Biosystems Inc. (Foster City, CA, USA).

2.2. Animals and experimental design

All procedures involving the use of live animals as described in this study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. The National Institutes of Health guidelines for humane treatment of animals were followed. Seven-week-old female Sprague-Dawley rats (ovariectomized at 4 weeks of age) were obtained from Harlan Sprague-Dawley

Laboratory, Houston, TX, USA. After arrival, they were allowed to acclimatize for a week before the start of the experimentation. The animals were housed in groups under controlled conditions of temperature (25 °C) and photoperiod (12-h light/12-h dark cycle), and had free access to food and water throughout the experimental period. The animals were weighed and then randomly divided into the following eight experimental groups: control (vehicle), ICI-182,780 (750 μg/rat), E_2 (500 μg/rat), 4-OH-E_2 (500 μg/rat), E_2 (500 μg/rat)+ICI-182,780 (750 μg/rat), 4-OH-E_2 (500 μg/rat)+ICI-182,780 (750 μg/rat), 4-MeO-E_2 (500 μg/rat), and 4-MeO-E_1 (500 μg/rat). All steroids were administered through oral gavage in 1% methylcellulose once daily for 7 consecutive days. The dosage and treatment time were selected with reference to earlier studies (Liu and Bachmann, 1998; Yamabe et al., 2010). Body weight and food intake were monitored once daily.

At the end of the experiment, the animals were fasted overnight before euthanasia with CO_2 overdose followed by decapitation. Blood samples (4–5 ml) were collected from each animal and serum samples were prepared. The uteri were removed, trimmed of excess connective tissues, and weighed. Gonadal white adipose tissues were also removed and weighed. Part of the liver was removed and snap-frozen in liquid nitrogen and kept at −80 °C until used for extraction of lipids or mRNAs. Part of the gonadal white adipose tissue and liver was placed in the 10% buffered formalin for fixation overnight for histological analysis.

2.3. Histological analysis of adipose tissue and liver

Fixed adipose tissue and liver were dehydrated in a graded ethanol-xylene series, and then embedded in paraffin and then sliced into 5-μm thick sections. The slides were deparaffinized with three changes of xylene and rehydrated through a graded ethanol-water series. Standard hematoxylin and eosin (H/E) staining procedure was used to stain the tissue sections. The slides were dehydrated in a graded ethanol-xylene series and then mounted for microscopic viewing and analysis.

2.4. Determination of serum cholesterol, triglyceride and lipoprotein profiles

After decapitation, trunk blood was collected, and serum was prepared and stored at −20 °C until used. Serum total cholesterol and triglyceride (TG) were measured by commercial colorimetric kits obtained from Wako Chemicals Inc. (Richmond, VA, USA) and Thermo Electron Co. (Pittsburgh, PA, USA), respectively, according to the manufacturer-recommended protocols.

Serum lipoprotein profiles were analyzed by fast protein liquid chromatography (FPLC) with a Suprose 6 column and phosphate buffered saline (PBS, pH=7.4) as the mobile phase (MacLean et al., 2003; Nijstad et al., 2011; Tohyama et al., 2009). Briefly, 300-μl serum sample (containing 60-μl taken from each of the five animals in the same treatment group) was applied to the column, and then fractions eluted off the column were collected. Each fraction was measured for total cholesterol and TG content using commercial kits as mentioned above.

2.5. Lipid extraction from liver

The lipids from liver tissue were extracted according to an earlier report (Folch et al., 1968). Briefly, liver tissue (100 mg) was homogenized and then 4 ml of methanol+butylated hydroxytoluene and 100 μl of the internal standard heptadecanoic acid (C17:0) were added into each tube and then vortexed briefly. Chloroform (8 ml) was added and the extraction tube was shaken overnight. The content was filtered through a filter paper (Whatman #1) into a clean extraction tube and then 1.6 ml of 1 M potassium chloride was added. The tube was vortexed and centrifuged for 5 min at 1000*g* and the bottom phase was transferred into another clean tube. The solvent was dried under a
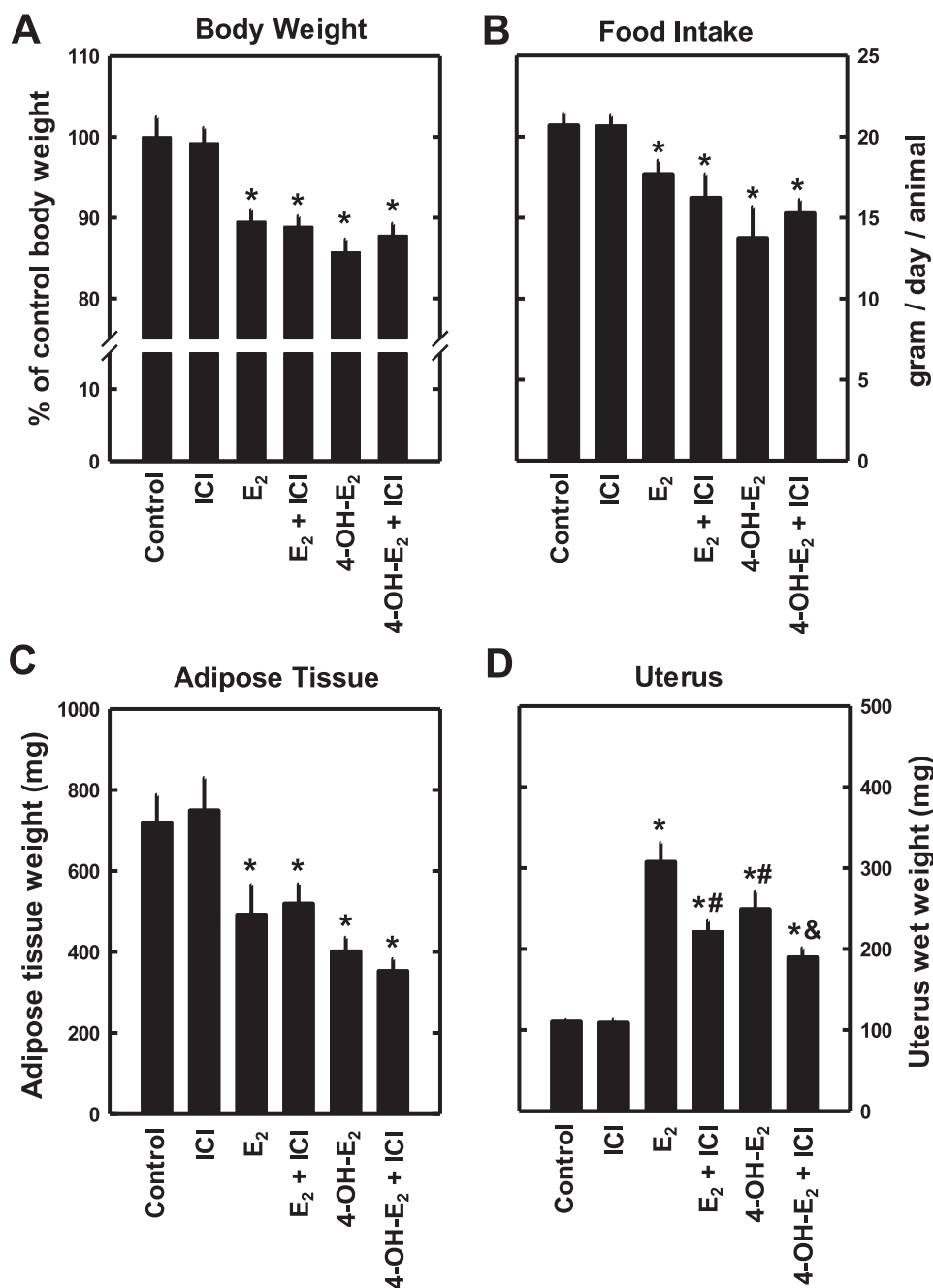
Fig. 2. The effect of estrogens on body weight (*A*), food intake (*B*), uterine wet weight (*C*), and gonadal adipose tissue weight (*D*) in OVX female Sprague-Dawley rats. The rats were treated orally with vehicle (control), E_2 (500 μg/rat), 4-OH-E_2 (500 μg/rat), E_2 (500 μg/rat)+ICI-182,780 (750 μg/rat), or 4-OH-E_2 (500 μg/rat)+ICI-182,780 (750 μg/rat) for 7 days. The body weights of rats in different treatment groups were expressed as a percentage of the average body weight of rats in the control group on day 7 of treatment. Each treatment group has 5 animals, and each data is the mean ± S.D. * $P < 0.05$ compared with the vehicle control; # $P < 0.05$ compared with the corresponding E_2 group; & $P < 0.05$ compared with the corresponding 4-OH-E_2 group. Abbreviation: ICI=ICI-182,780.

stream of nitrogen gas. The lipid pellet was dissolved either in dichloromethane as sample preparation for gas chromatography (GC; described below) or in 10% Triton X-100 in isopropanol for measurement of cholesterol and TG using the commercial kits.

2.6. Detection of various fatty acids associated with triglyceride and cholesterol esters

Aliquots (100 μl) of the dichloromethane solution of lipids were spotted on a thin layer chromatography (TLC) plate for separation using a solvent system containing hexane/ether/acetic acid (79/20/1; *v/v/v*) (Xu et al., 2011). The bands containing triglyceride (TG) or cholesterol esters (CEs) were individually scrapped off and transferred to a 15-ml

tube. A mixture containing boron trifluoride (BF_3) (0.5 ml)+benzene (0.4 ml)+methanol (1.1 ml) for TG, or containing BF_3 (0.7 ml)+benzene (0.6 ml)+methanol (0.7 ml) for CEs, were added to the tubes, which were then placed in a dry bath at 100 ºC for 30 min (TG) or 45 min (CEs). Afterwards, tubes were cooled on ice and 2 ml water+4 ml pentane was added. The tubes were vortexed and centrifuged for 5 min at 100*g*. The upper phase (pentane) was transferred to a new tube and solvents were dried under a stream of nitrogen gas. Dichloromethane (60 μl) was added to dissolve the fatty acid methyl esters (FAMEs) and the samples were injected into GC. The SP-2560 column (obtained from Supelco through Sigma-Aldrich, Co.) was used to separate individual FAMEs. The conditions of GC and the flame ionization detector (FID) were set according to an earlier report (Xu et al., 2011).

2.7. Real-time PCR analysis of liver tissue

The messenger RNAs were extracted from the animal livers using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration of each sample was determined by spectrophotometric analysis at 260 nm. The integrity of each RNA sample was evaluated using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA synthesis was performed with 1 µg of total RNAs in 20 µl using random primers (Invitrogen) and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). PCR analysis was performed on a 7500 Real-Time PCR System (Applied Biosystems). Reactions were performed in a 25-µl volume containing 12.5 µl of 2× SYBR Green PCR Master Mix (Applied Biosystems), 1 µl of cDNA (corresponding to 25 ng of reverse-transcribed total RNAs), and 5 pmol of each primer. After an initial incubation for 2 min at 50 °C, the cDNA was denatured at 95 °C for 10 min followed by 40 cycles of PCR (95 °C, 15 s, 60 °C, 60 s). Data analyses were performed on the 7500 System SDS software (version 1.3.1; Applied Biosystems). All samples were normalized according to the respective expression levels of β-actin. The real-time PCR reaction was carried out with the SYBR Green PCR Master Mix. The primer sequences for the target genes were as follows: for β-actin, 5′-GGCATCCATGAAACTACATT-3′ and 5′-GATCTTCATGGTGCTAGGAG-3′; for LXRα, 5′-GTGAGAGCATCA CCTTCCTC-3′ and 5′-CTCTACTTGGAGCTGGTCCT-3′; for PPARγ, 5′-CTCTGTGGACCTCTCTGTGA-3′ and 5′-GTGGCCTGTTGTAGAGT TGG-3′; for Leptin, 5′-GGTCACCGGTTTGGACTTCA-3′ and 5′-GGTCTGGTCCATCTTGGACAA-3′; and for CYP7A1, 5′-GCAGCCTCTG AAGAAGTGAG-3′ and 5′-GAAGTCCTCCTTAGCTGTGC-3′.

2.8. Statistical analysis

Most of the quantitative data are expressed as means ± S.D. The one-way analysis of variance (*ANOVA*) followed by *post-hoc* Turkey's test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of 4-OH-E₂ and E₂ on animal body weight, food intake and adipose tissue weight

The body weights of OVX female Sprague-Dawley rats in different groups before and after treatment (*i.e.*, on day 0 and day 7) were shown in Supplementary Table 1. For comparison, the body weights of rats in different treatment groups (Fig. 2A) were presented as a percentage of the body weight of the control group animals on day 7. We found that rats treated with 4-OH-E₂ or E₂ had a significantly lower body weight compared to rats treated with vehicle (Fig. 2A). Notably, 4-OH-E₂ appears to have a stronger body weight-reducing effect than E₂, although the difference is not statistically significant ($P=0.087$). Treatment of animals with ICI-182,780 did not appreciably affect the body weight. The body weights of rats treated with E₂ or 4-OH-E₂ in combination with ICI-182,780 were not significantly different from those rats treated with E₂ or 4-OH-E₂ alone (Fig. 2A). In Fig. S1, we also showed the relative body weight change of rats after treatment (on day 7) compared with the body weights before treatment (on day 0). Rats receiving vehicle or ICI-182,780 treatment had approximately 12% increase in their average body weights during the 7-day period, but rats treated with 4-OH-E₂ or E₂ did not have appreciable body weight gains (Fig. S1).

The average food intake per animal was measured daily at the same time (prior to measuring the body weight) during the 7-day treatment period. ICI-182,780 alone had no effect on animal food intake. Rats treated with 4-OH-E₂ and E₂ consumed 13.7 ± 1.9 and 17.7 ± 0.8 g/ animal/day, respectively, which is less than the average daily intake by rats in the control group (20.7 ± 0.8 g/animal/day) (Fig. 2B). Notably,

4-OH-E₂ caused a slightly bigger reduction in food intake than did E₂ ($P=0.071$). Animals treated with 4-OH-E₂+ICI-182,780 consumed 15.3 ± 0.8 g/animal/day, and those treated with E₂+ICI-182,780 consumed 16.2 ± 1.5 g/animal/day, which are not significantly different from the animals treated with 4-OH-E₂ or E₂ alone (Fig. 2B).

Gonadal adipose tissue weight was decreased markedly in animals treated with 4-OH-E₂ and E₂ (Fig. 2C). Treatment with 4-OH-E₂ resulted in a slightly bigger decrease in adipose tissue weight than did E₂ ($P=0.302$). Co-treatment with ICI-182,780 did not significantly affect the adipose tissue weight change caused by 4-OH-E₂ or E₂ treatment.

4-OH-E₂ had a weaker estrogenic effect than E₂ in the uterus, a representative estrogen target organ (Fig. 2D). Furthermore, co-treatment with ICI-182,780 significantly reduced uterine weight increase compared to animals treated with 4-OH-E₂ or E₂ alone, indicating that ICI-182,780 was absorbed into the circulation and functionally active as an ER antagonist in the uterus.

Histological analysis of the adipose tissue showed that treatment of animals with 4-OH-E₂ or E₂ resulted in marked reduction in adipocyte size (compare Fig. 3C and D with Fig. 3A). It appeared that after estrogen treatment, the adipocyte size became less uniform than what was seen in the control animals. Treatment with ICI-182,780 alone had no effect on the adipocyte size (Fig. 3B). Co-treatment with ICI-182,780 slightly diminished the effect of 4-OH-E₂ and E₂ on adipocyte size (compare Fig. 3E and F with Fig. 3C and D as well as the quantitative data in Fig. 3G). Histology of liver tissues from estrogen-treated animals revealed no notable signs of hepatotoxicity (data not shown).

3.2. Effect of 4-OH-E₂ and E₂ on serum cholesterol level

Treatment of animals with 4-OH-E₂ or E₂ strongly decreased the serum cholesterol level by 94.1% or 56.8%, respectively (Fig. 4A). It is apparent that 4-OH-E₂ had a markedly stronger effect than E₂ in reducing the serum cholesterol level. Co-treatment with ICI-182,780 slightly reduced 4-OH-E₂'s effect on serum cholesterol level, but it did not alter E₂'s effect. Serum TG level was decreased by treatment with 4-OH-E₂ or E₂, and combined treatment with ICI-182,780 did not alter their effect (Fig. 4B).

Gel filtration method is commonly used to fractionate serum lipoproteins according to their sizes. In agreement with reports in the literature (Nijstad et al., 2011; Tohyama et al., 2009), we found that the major cholesterol peak in rat serum was associated with HDL, namely, the HDL-cholesterol (Fig. 4C). Treatment of animals with E₂ and 4-OH-E₂ decreased the HDL-cholesterol level, and 4-OH-E₂ had a markedly stronger effect than E₂ in this respect (Fig. 4C and D). Co-treatment with ICI-182,780 partially reduced 4-OH-E₂'s modulating effect on the HDL-cholesterol level (Fig. 4D). The major serum TG peaks were free TG and VLDL-TG (Fig. 4E). 4-OH-E₂ and E₂ decreased the levels of VLDL-TG. Combined treatment with ICI-182,780 increased the VLDL-TG level compared with 4-OH-E₂ treatment alone (Fig. 4F). The effect of 4-OH-E₂ and E₂ on free TG level was not as prominent as their effect on VLDL-TG level (Fig. 4G). Intriguingly, treatment with ICI-182,780 alone also reduced, to some extent, the serum VLDL-TG and free TG levels compared to vehicle-treated control group.

3.3. Effect of 4-OH-E₂ and E₂ on liver cholesterol composition and level

In the liver, the total TG content was approximately 4 times higher than the total cholesterol content (Fig. 5A and B). 4-OH-E₂ markedly decreased liver TG level but E₂ did not have this effect. Combined treatment with ICI-182,780 did not alter the effect of 4-OH-E₂ on liver TG level (Fig. 5A). Treatment of animals with 4-OH-E₂ or E₂ alone or in combination with ICI-182,780 did not significantly affect liver cholesterol level (Fig. 5B).
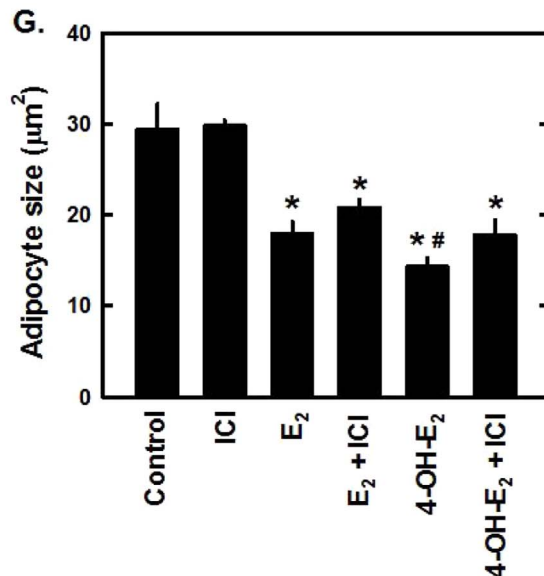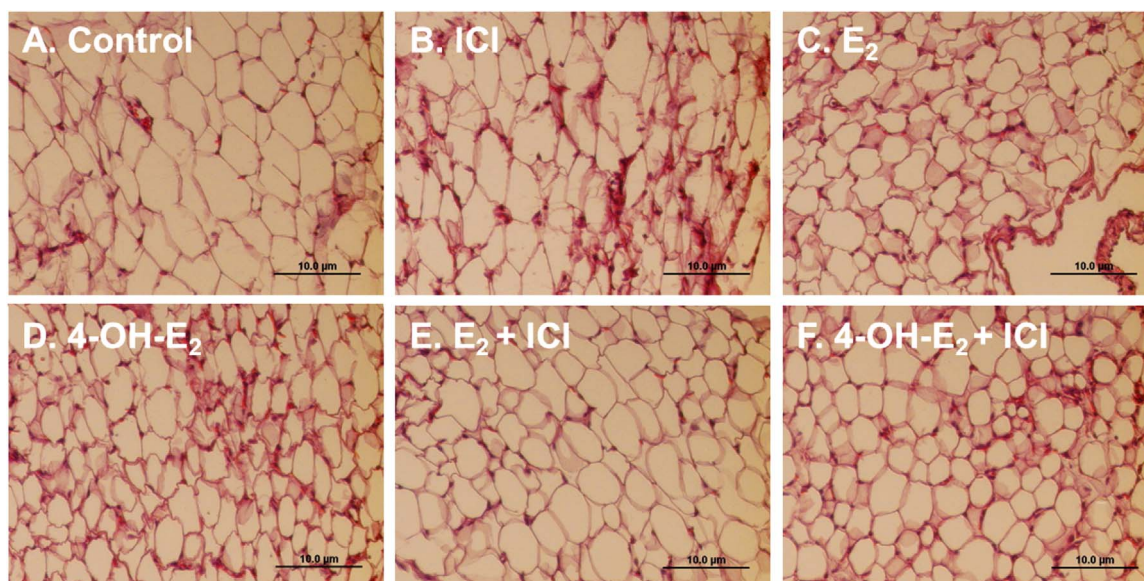
Fig. 3. H/E staining of the gonadal adipose tissue obtained from rats treated with vehicle (*A*), ICI -182,780 (*B*), E_2 (*C*), 4-OH-E_2 (*D*), E_2+ICI-182,780 (*E*), or 4-OH-E_2+ICI-182,780 (*F*). The pictures were taken at 200× magnification. (*G*) The adipocyte size of animals treated with different estrogens or in combination with ICI-182,780. Each group had 5 animals, and each data is the mean ± S.D. * $P < 0.05$ compared with the vehicle control; # $P < 0.05$ compared with the corresponding E_2 group; & $P < 0.05$ compared with the corresponding 4-OH-E_2 group. Abbreviation: ICI=ICI-182,780.

Liver TG and cholesterol esters (CEs) were extracted and separated by TLC. Transmethylation method was used to substitute glycerol or cholesterol in TG and CEs with a methyl group, and the methyl esters of various endogenous fatty acids were analyzed using GC-FID (data shown in Figs. 5C and D, and S2A, B). Notably, the majority of fatty acids was found to be present in the form of TG, but not CEs. In TG, the most abundant fatty acids detected were C18:2n6c (linoleic acid, ω-6 fatty acid), C16:0 (palmitic acid), and C18:1n9c (oleic acid) (Fig. 5C). Almost all fatty acids associated with TG were uniformly decreased by treatment with 4-OH-E_2 or E_2. 4-OH-E_2 caused a stronger decrease in the levels of C16:0, C18:1n9c, C18:2n6c, C18:3n3 and C20:4n6 compared with E_2 (Figs. 5C and S2A). Co-treatment with ICI-182,780 did not significantly alter the effect of 4-OH-E_2 or E_2 on the fatty acid content. However, it appeared that combined treatment of ICI-182,780 and 4-OH-E_2 caused a further decrease in TG-associated fatty acid content although there was no significant difference between the levels of fatty acids in these two groups of animals.

The most abundant fatty acids contained in hepatic CEs were C18:1n9c (oleic acid) and C18:2n6c (linoleic acid, ω-6 fatty acid) (Fig. 5D). E_2 had no effect on the levels of these two fatty acids. However, 4-OH-E_2 decreased the levels of these two fatty acids, and its effect was not altered by co-treatment with ICI-182,780. Palmitoleic acid (C16:1) level was also reduced by 4-OH-E_2 but not by E_2 treatment (Fig. S2B). For saturated fatty acids palmitic acid (C16:0) and stearic acid (C18:0), co-treatment with ICI-182,780 restored their reduced levels caused by 4-OH-E_2 or E_2 treatment (Figs. 5D and S2B). In addition, while E_2 increased the CE-associated C18:3n3 level, 4-OH-E_2 did not have a similar effect. Co-treatment with ICI-182,780 reduced C18:3n3 levels in animals treated with 4-OH-E_2 or E_2 (Fig. S2B).
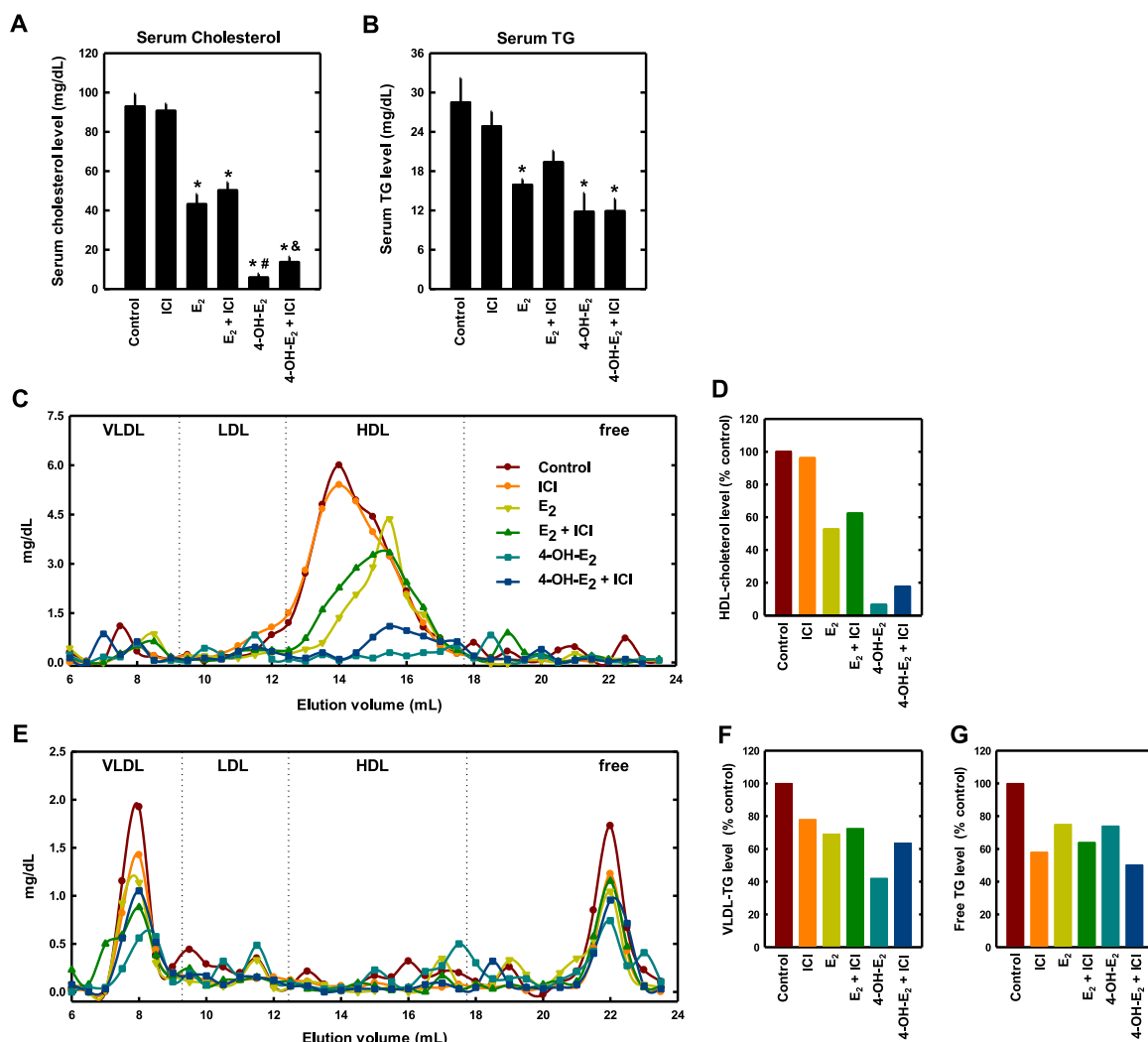
Fig. 4. Serum cholesterol and TG levels in OVX female Sprague-Dawley rats treated orally with an estrogen alone or in combination with ICI-182,780 (ICI) for 7 days. Levels of serum total cholesterol (A) and TG (B) were measured using colorimetric kits. Each group had 5 animals, and each data in (A) and (B) is the mean ± S.D. * $P < 0.05$ compared with the vehicle control; # $P < 0.05$ compared with the corresponding E_2 group; & $P < 0.05$ compared with the corresponding 4-OH-E_2 group. The serum samples from all animals in the same treatment group were pooled together prior to the lipoprotein fractionation using FPLC. The cholesterol (C) and TG (E) levels in each fraction were measured using commercial colorimetric kits. The areas under the curves for HDL cholesterol (D), VLDL TG (F) and free TG (G) for each treatment group were calculated and normalized to that of the control group.

3.4. Effect of 4-OH-E_2 and E_2 on hepatic expression of CYP7A1, LXRα, leptin and PPARγ

Liver is the most important organ in regulating lipid homeostasis. To probe the underlying mechanism by which 4-OH-E_2 and E_2 regulate cholesterol and TG synthesis and secretion, we determined the expression levels of a number of key lipid regulators in the liver. As shown in Fig. 6A–D, CYP7A1 was induced by 3.3- and 9.3-fold by E_2 and 4-OH-E_2 treatment, respectively. Interestingly, while co-treatment of E_2+ICI-182,780 resulted in a higher induction of CYP7A1 compared with E_2 alone, co-treatment of 4-OH-E_2+ICI-182,780 resulted in a lower induction of CYP7A1 compared with 4-OH-E_2 alone. Leptin was induced by 2.3- and 1.8-fold by 4-OH-E_2 and E_2, respectively. Combined treatment with ICI-182,780 led to higher induction of leptin compared with 4-OH-E_2 or E_2 alone. LXRα was induced by 2.0- and 3.3-fold by 4-OH-E_2 and E_2, respectively, and combined treatment with ICI-182,780 did not affect the induction. The change in PPARγ levels was rather modest, with 1.7- and 1.9-fold increase by 4-OH-E_2 and E_2, respectively. However, there was no significant difference in PPARγ level between the control group and the treatment groups.

3.5. Lipid-modulating effects of 4-MeO-E_2 and 4-MeO-E_1

Rats treated with 4-MeO-E_2 and 4-MeO-E_1 had significantly lower body weights compared to those treated with vehicle (Fig. 7A). Notably, 4-MeO-E_2 and 4-MeO-E_1 at the same dose (500 µg/rat) had a comparable body weight-reducing effect as 4-OH-E_2 (Fig. 7A). 4-MeO-E_2 and 4-MeO-E_1 treatment also decreased the food intake and adipose tissue weight in rats to a similar extent as did 4-OH-E_2 (Fig. 7B and C). 4-MeO-E_2 and 4-MeO-E_1 treatment also decreased adipocyte size (Fig. 8). In contrast, the uterotrophic activity of 4-MeO-E_2 and 4-MeO-E_1 even at the high dose (500 µg/rat) was still significantly lower than that of 4-OH-E_2 (Fig. 7D).

Furthermore, 4-MeO-E_2 and 4-MeO-E_1 at the same dose as 4-OH-E_2 strongly reduced serum cholesterol and TG levels (Fig. 9). The effect of 4-MeO-E_2 and 4-MeO-E_1 on serum cholesterol was weaker than 4-OH-E_2 (P=0.021, 0.048, respectively). In the serum lipoprotein profiles, 4-MeO-E_2 and 4-MeO-E_1 strongly decreased serum HDL-cholesterol level, although their effect was slightly weaker than that of 4-OH-E_2 (Fig. 9D). Similarly, the decrease in serum VLDL-TG by 4-MeO-E_2 or 4-MeO-E_1 was also weaker than what was seen with 4-OH-E_2 (Fig. 9F). The effect of 4-MeO-E_2 and 4-MeO-E_1 on serum free TG levels was not as prominent as their effect on VLDL-TG levels (Fig. 9G).
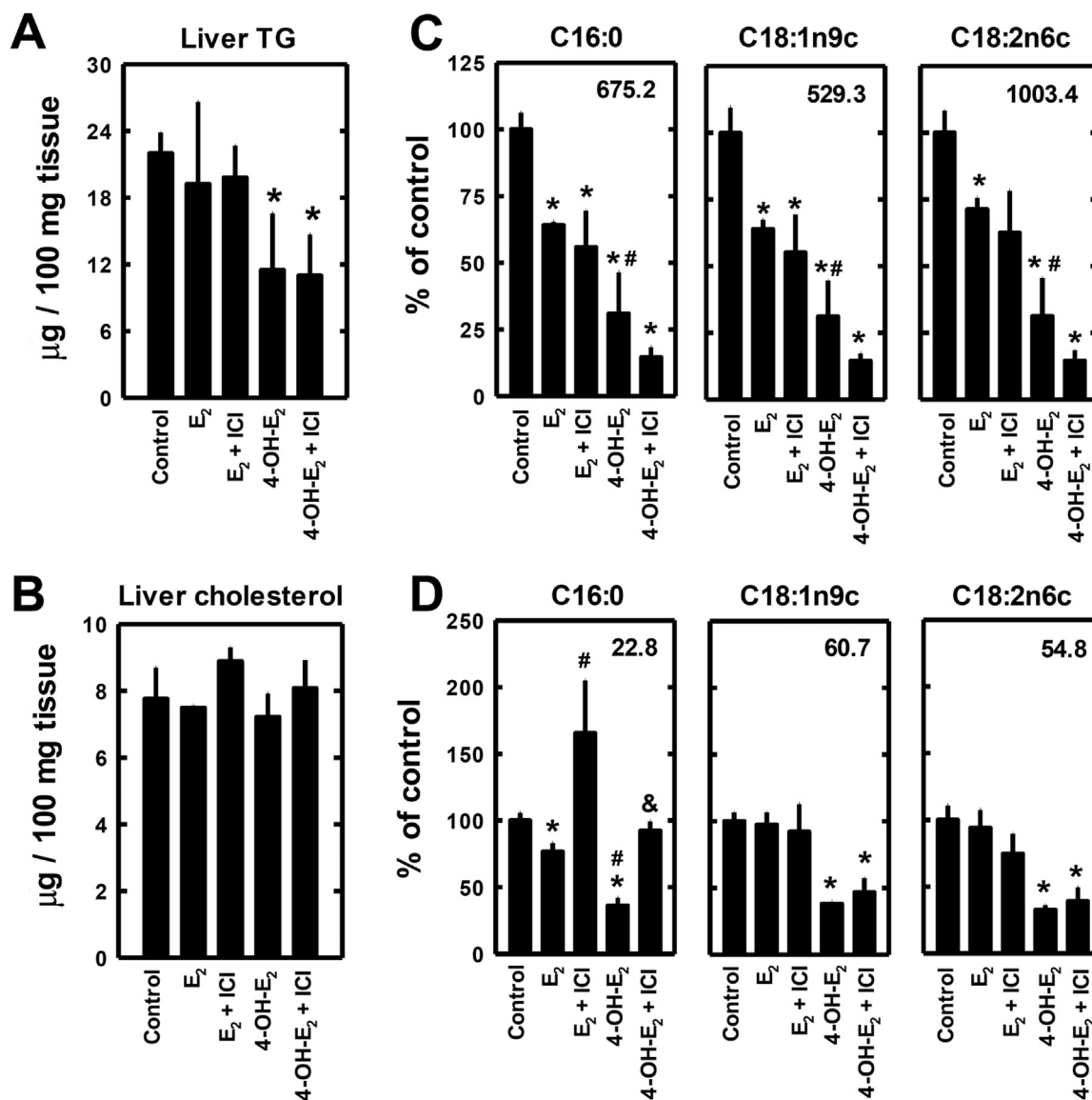
Fig. 5. Levels of liver TG (*A*), cholesterol (*B*) and the fatty acid composition of TG (*C*) and CEs (*D*) in OVX female Sprague-Dawley rats treated orally with estrogen alone or in combination with ICI-182,780 (ICI) for 7 days. The fatty acid composition was determined using GC-FID with C17 as internal standard for each sample (*C*) and (*D*). Each fatty acid in the control group was set as 100% and the values of fatty acid amount (pg/ mg tissue) in the control group were shown inside each panel of (*C*) and (*D*). Each group had 5 animals, and each data is the mean ± S.D. * $P < 0.05$ compared with the vehicle control; # $P < 0.05$ compared with the corresponding E_2 group; & $P < 0.05$ compared with the corresponding 4-OH-E_2 group.

4. Discussion

Obesity is the most common cause for the development of metabolic syndrome and cardiovascular diseases (Moller and Kaufman, 2005). In developed countries such as the U.S., over 60% of adults exceed the normal range of body mass index, a medical indicator for being obese (Friedman, 2004; Haslam and James, 2005; Lazar, 2005; Yach et al., 2006). In support of the important etiological role of obesity in cardiovascular diseases, clinical studies have shown that a reduction in body weight by even 5% helps significantly decrease blood pressure in hypertensive patients (Poirier et al., 2006). The strong correlation between the extent of weight loss and its health benefits makes it hugely important to develop means that can effectively reduce excess body weight in a safe and sustainable fashion.

The results of our present study show that 4-OH-E_2 had a markedly stronger effect than E_2 in decreasing the gonadal adipocyte size and blood cholesterol level. However, the estrogenic activity in the uterus (a representative estrogen target organ) of 4-OH-E_2 is weaker than E_2. It is well documented that 4-OH-E_2 has a relatively lower estrogenic

activity *in vivo* than E_2 because of the following two main reasons: (i) 4-OH-E_2 has a lower binding affinity for ERs than E_2 (approximately 70% of E_2 (Markides and Liehr, 2005)), and (ii) 4-OH-E_2 also has a significantly faster rate of metabolic disposition *in vivo* (due to rapid *O*-methylation catalyzed by catechol-*O*-methyltransferase) (Ball and Knuppen, 1990). This apparent discrepancy suggests that some of the lipid-modulating effects of E_2 and 4-OH-E_2 are not mediated by the known ERs − if it were, then E_2 is expected to have a uniformly stronger effect than 4-OH-E_2 in exerting all these effects.

To provide further support for the above suggestion, we used ICI-182,780, which is a pure antagonist for both ERα and ERβ and is different from tamoxifen (an ER partial agonist) (Howell et al., 2000). As shown in Fig. 2D, administration of ICI-182,780 at a comparable molar dose of 4-OH-E_2 and E_2 inhibits the uterotropic activity of both estrogens by approximately 40%. However, the body weight (Fig. 2A), food intake (Fig. 2B), adipose tissue weight (Fig. 2C), and liver TG level (Fig. 5A) in animals treated with 4-OH-E_2 or E_2 are not significantly altered by co-treatment with ICI-182,780. This lack of effect is not due to the inadequacy of the ICI-182,780 dose used because it significantly
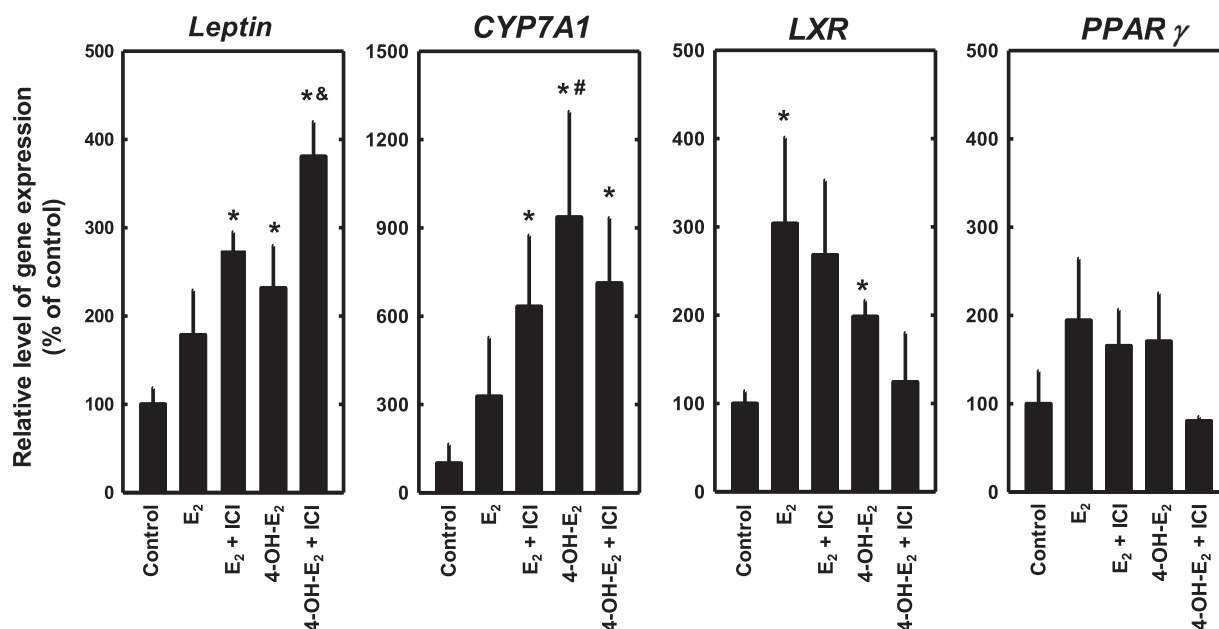
Fig. 6. Real-time PCR detection of gene expression levels in the liver of OVX female Sprague-Dawley rats treated orally with an estrogen alone or in combination with ICI-182,780 (ICI) for 7 days. Each gene expression level is normalized with β-actin and the level of gene expression in the control group is set as 100%. Each group has 5 animals, and each data is the mean ± S.D. * $P < 0.05$ compared with the vehicle control; # $P < 0.05$ compared with the corresponding E_2 group; & $P < 0.05$ compared with the corresponding 4-OH-E_2 group.
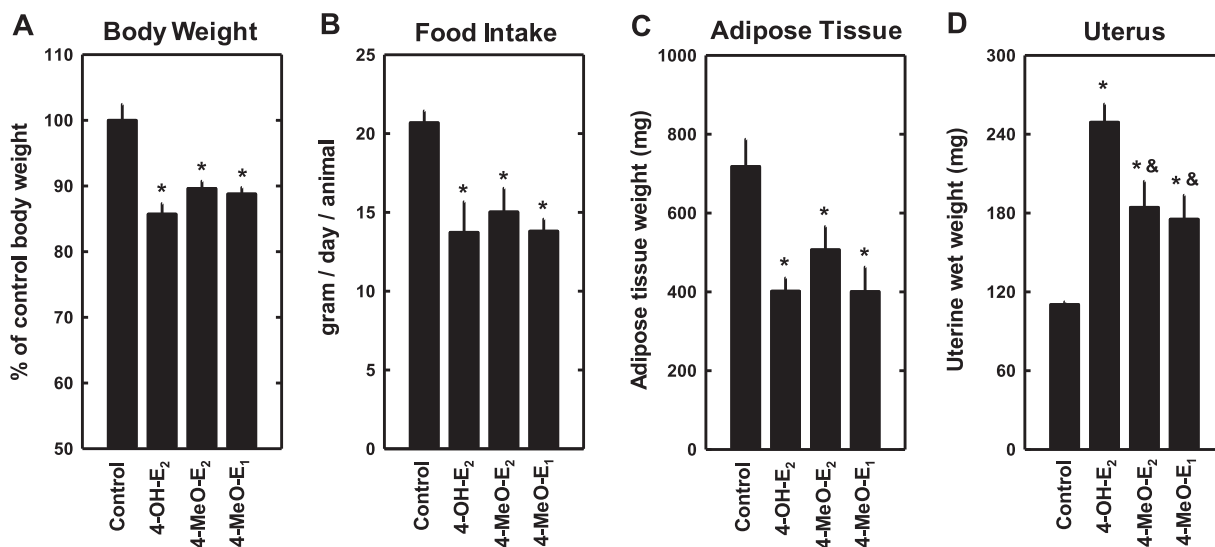

Fig. 7. Effect of estrogens on body weight (A), food intake (B), uterine wet weight (C), and gonadal adipose tissue weight (D) in OVX female Sprague-Dawley rats. The rats were treated orally with vehicle (control), 4-OH-E_2 (500 μg/rat), 4-MeO-E_2 (500 μg/rat), or 4-MeO-E_1 (500 μg/rat) for 7 days. The body weights of rats in different treatment groups were expressed as a percentage of the average body weight of rats in the control group on Day 7 of treatment. Each group had 5 animals, and each data is the mean ± S.D. * $P < 0.05$ compared with the vehicle control; & $P < 0.05$ compared with the corresponding 4-OH-E_2 group. Note that the data for the control and 4-OH-E_2 groups are the same as those shown in Fig. 2, and they are included for ease of comparison. Abbreviation: ICI=ICI-182,780.

reduces these estrogens' hormonal activity in the uterus, which is a representative estrogen target organ where the estrogen actions are known to be mediated by ERs. Hence, these results provide additional support for the suggestion that the effects of estrogens on body weight, serum cholesterol level and liver TG are mostly not mediated by the ER-dependent pathways.

Although 4-OH-E_2 may exert several lipid-modulating effects independently of the ER pathways, it is also apparent that the ERs are involved in mediating some of the other observed effects. For instance, the adipocyte size appears to be regulated, to some extent, by the ER-mediated pathways because co-treatment with ICI-182,780 significantly reduces the effect of 4-OH-E_2 on adipocyte size whereas ICI-182,780 treatment alone has no effect. Similarly, the observation that animals treated with 4-OH-E_2+ICI-182,780 have a small but

significant increase in serum cholesterol level compared with animals treated with 4-OH-E_2 alone suggests that the estrogen's effect on serum cholesterol is partly mediated by the ERs (Fig. 4A). Also, the hepatic levels of saturated fatty acids appear to be also associated with the ER-dependent pathways (Figs. 5D and S2B). The underlying mechanism as to how ERs regulate these processes is not clear at present.

The results of our present study show that 4-OH-E_2 is a strong regulator of hepatic CYP7A1 expression (Fig. 6). CYP7A1 gene is a well-known LXRα target gene (Peet et al., 1998; Tontonoz and Mangelsdorf, 2003). Since CYP7A1 is the rate-limiting enzyme in hepatic bile biosynthesis (Peet et al., 1998), its induction may result in accelerated cholesterol clearance and thus may partly contribute to the drastic reduction in serum cholesterol level as observed in this study. Interestingly, this induction of CYP7A1 by 4-OH-E_2 and E_2 likely
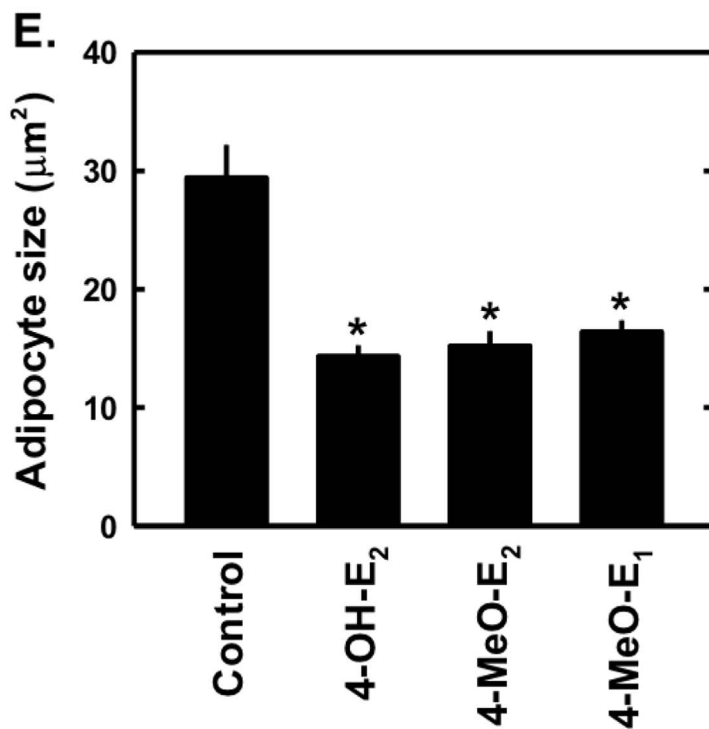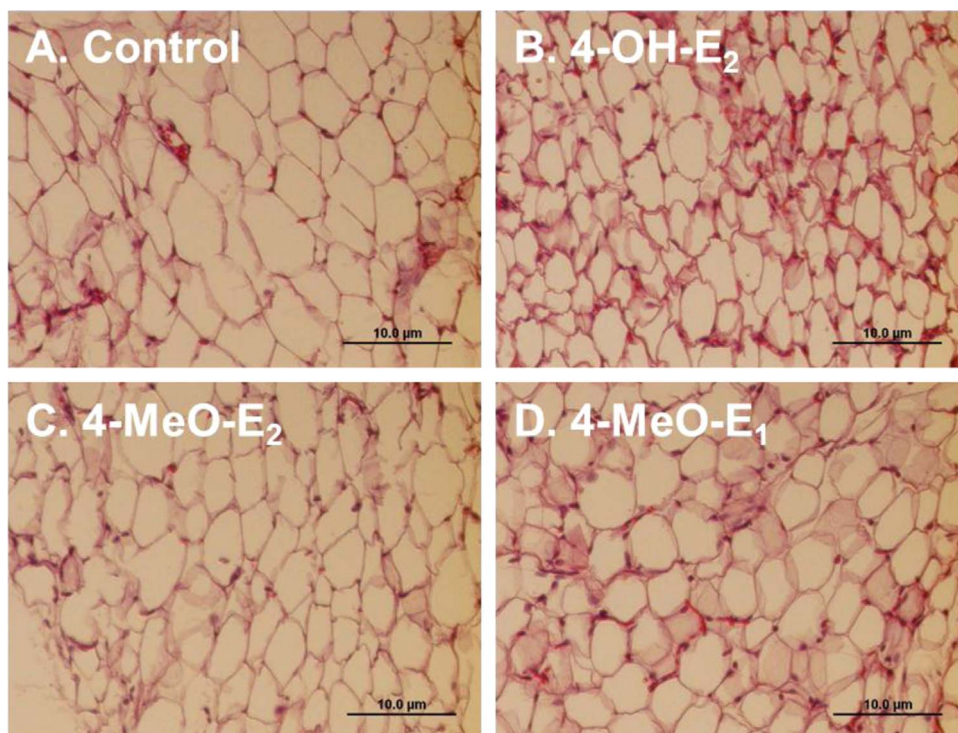
Fig. 8. H/E staining of the adipose tissue obtained from rats treated with vehicle (*A*), 4-OH-E$_2$ (*B*), 4-MeO-E$_2$ (*C*), 4-MeO-E$_1$ (*D*). The pictures were taken at 200× magnification. (*E*) Adipocyte sizes of rats treated with different estrogens. Each group had 5 animals, and each data is the mean ± S.D. * $P < 0.05$ compared with the vehicle control.

is not mediated by ERs since the induction is not affected by co-treatment with ICI-182,780. The exact mechanism by which estrogens elicit CYP7A1 induction merits further investigation.

In contrast to our results showing that estrogens cause CYP7A1 induction and LXRα activation in liver, it has also been reported that E$_2$ can inhibit LXR activation through direct binding of the ERα to LXR in liver and thereby reduces liver TG levels (Han et al., 2014). This inconsistency may be due to the different animal models used.

Leptin is a peptide hormone that informs the brain of the whole body energy reserve status. This pathway has been quite extensively studied (discussed in Ahima and Osei, 2004). It has been reported that women have higher serum leptin levels than men in both lean and obese populations (Saad et al., 1997). Estrogens have been suggested to play a role in regulating serum leptin as well as leptin mRNA levels in adipose tissues (Ahrens et al., 2014; Xu et al., 2012). It has been reported that serum leptin levels correlated with liver leptin mRNA
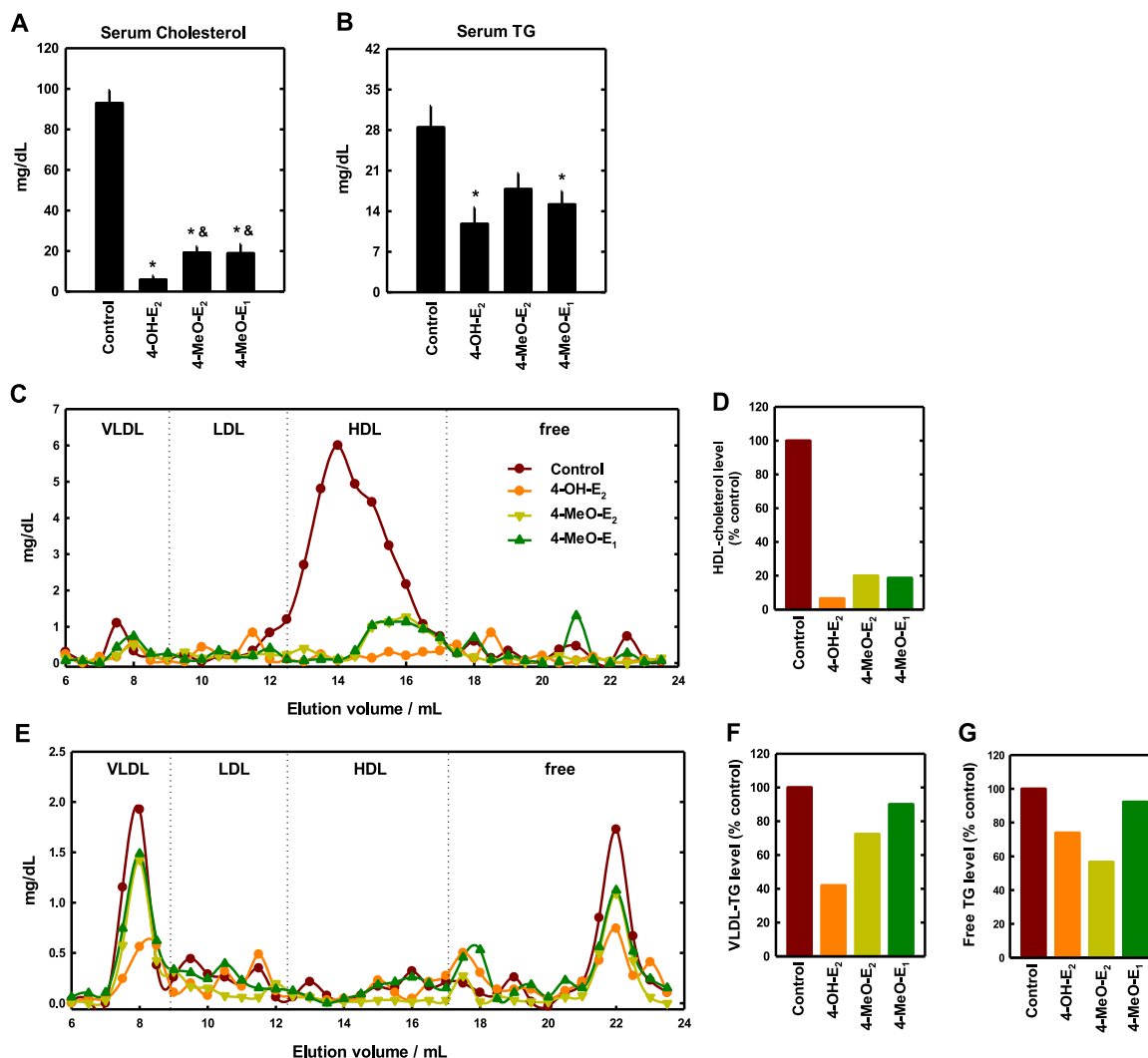
Fig. 9. Serum cholesterol and TG levels in OVX female Sprague-Dawley rats treated orally with control (vehicle), 4-OH-E_2 (500 µg/rat), 4-MeO-E_2 (500 µg/rat), 4-MeO-E_1 (500 µg/rat) for seven days. Levels of serum total cholesterol (*A*) and TG (*B*) were measured using colorimetric kits. Each group had 5 animals, and each data in (*A*) and (*B*) is the mean ± S.D. * $P <$ 0.05 compared with the vehicle control; [#] $P < 0.05$ compared with the corresponding E_2 group; [&] $P < 0.05$ compared with the corresponding 4-OH-E_2 group. The serum samples from all animals in the same treatment group were pooled together prior to the lipoprotein fractionation using FPLC. The cholesterol (*C*) and TG (*E*) levels in each fraction were measured using the commercial colorimetric kits. The areas under the curve for HDL cholesterol (*D*), VLDL TG (*F*) and free TG (*G*) for each treatment group were calculated and normalized to that of the control group. Note that the data for the control and 4-OH-E_2 groups are the same as those shown in Fig. 4, and they are included for ease of comparison.

levels (Wang et al., 2015). Thus, the elevated levels of leptin mRNA in liver in animals treated with 4-OH-E_2 or E_2 probably lead to the increased levels of serum leptin, which may contribute to the reduced food intake of these animals. However, the mechanism of this regulation remains to be elucidated since our results show that combined treatment with ICI-182,780 does not suppress the effect of 4-OH-E_2 or E_2, suggesting that this regulatory effect of estrogen likely is not mediated by ERs.

PPARγ is known to be involved in regulating many genes involved in adipocyte differentiation and adipocyte lipid storage (Christodoulides and Vidal-Puig, 2010). Induction of PPARγ is usually associated with increase in adipose tissue weight (Christodoulides and Vidal-Puig, 2010). However, we find that the adipose tissue weight and adipocyte size are significantly reduced by estrogen administration. The role of PPARγ in estrogen-induced disorders of lipid metabolism is not clear at present.

In addition to testing the lipid-modulating effects of 4-OH-E_2 with its parent hormone E_2, we have also tested for comparison the effect of 4-MeO-E_2 and 4-MeO-E_1, two major endogenous metabolites of 4-OH-E_2 (Fig. 1). These two estrogen metabolites have little or no binding affinity for human ERα and ERβ (Zhu and Conney, 1998). We find that

these two 4-methoxyestrogen derivatives have similar lipid-modulating effects as 4-OH-E_2 (Figs. 7–9). This observation provides further support for the concept that some of the lipid-modulating effects of estrogens are not mediated by the ER-dependent signaling pathways. Further, it is suggested that the lipid-modulating effects of 4-OH-E_2 and E_2 *in vivo* may be partly contributed by their metabolic derivatives 4-MeO-E_2 and 4-MeO-E_1.

This possibility may be of particular significance in men because these two derivatives have strong lipid-modulating effect but very weak estrogenic hormonal activity. In support of this intriguing possibility, it is of note that the total daily amount of urinary excretion of 4-MeO-E_2 and 4-MeO-E_1 in men is surprisingly quite high (almost comparable to the amount present in the urine samples from premenopausal women) despite the fact that the circulating levels of the unmetabolized E_2 are usually very low in men (Kim et al., 2014). This observation may suggest that 4-MeO-E_2 and 4-MeO-E_1 are two endogenously-formed, hormonally-inactive estrogen derivatives in men that have a strong lipid-modulating function. From a pharmacological point of view, these two methylated estrogen derivatives would also be better drug candidates than E_2 or E-OH-E_2 as lipid-modulating agents, because of their markedly-reduced estrogenic activity. Moreover, 4-OH-E_2 is potentially

involved in tumor formation because it can induce DNA damage by forming DNA adducts (Ke et al., 2015; Yager, 2015).

5. Conclusion

In summary, the results of this study show that 4-OH-E$_2$ has a stronger effect in decreasing the adipocyte size, serum cholesterol level than does E$_2$. Moreover, most of the effects are not blocked by combined treatment with ICI-182,780, a pure ER antagonist. Interestingly, two methoxy-estrogen metabolites, 4-MeO-E$_2$ and 4-MeO-E$_1$, which have little binding affinity for human ERα and ERβ (Zhu and Conney, 1998; Zhu et al., 2006), were found to have a similar modulating effect on lipid profiles as 4-OH-E$_2$. The expression of hepatic CYP7A1 and leptin genes is markedly increased in rats treated with 4-OH-E$_2$ or E$_2$, and their induction is essentially not antagonized by co-treatment with ICI-182,780. These findings indicate that there may exist a novel ER-independent pathway that mediates some of the lipid-modulating effects of estrogens. The hormonally-weaker methoxy-estrogens may serve as better candidates as lipid-modulating agents in both genders of human subjects.

Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2017.02.032.

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