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

Pan Wang[a,b], Bao-Ting Zhu[a,c,*]

[a] Department of Pharmacology, Toxicology and Therapeutics, School of Medicine, The University of Kansas Medical Center, Kansas City, KS 66160, USA
[b] Laboratory of Molecular Toxicology, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[c] School of Science and Engineering, The Chinese University of Hong Kong, Shenzhen, Shenzhen, Guangdong 518172, China

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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 physiological 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

* Corresponding author at: School of Science and Engineering, The Chinese University of Hong Kong, Shenzhen, 2001 Longxiang Road, Longgang District, Shenzhen, Guangdong 518172, China.
E-mail address: BTZhu@CUHK.edu.cn (B.-T. Zhu).

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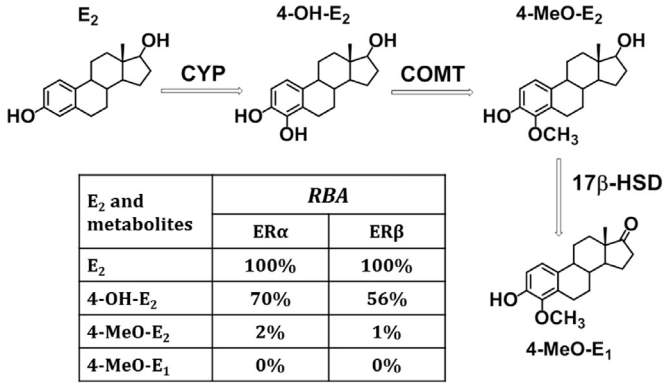

E_2 and metabolites	*RBA*	
	ERα	ERβ
E_2	100%	100%
4-OH-E_2	70%	56%
4-MeO-E_2	2%	1%
4-MeO-E_1	0%	0%

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