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17β-estradiol Prevents the Expression of CEBPα-mediated Adipocyte Marker Genes in Female Ovariectomized C57BL/6 Mice

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Adipogenesis is a complex sequence of events that culminates in the differentiation of fibroblast-like preadipocytes into specialized lipid-filled adipocytes and also involves a cascade of expression of many transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs). PPARγ and C/EBPs transcriptionally transactivate adipocyte specific genes, including fatty acid transport protein (FAT/CD36) and leptin. To determine whether 17β-estradiol modulates C/EBPα actions on adipogenesis in high fat diet-fed female ovariectomized (OVX) C57BL/6 mice, mice were treated with 17β-estradiol for 7 days and the effects of 17β-estradiol on adipose tissue mass and expression of adipocyte specific gene as well as C/EBPα were measured. Compared to vehicle-treated OVX control mice, OVX mice treated with 17β-estradiol for 7 days had lower adipose tissue weights that were similar to weights in high fat diet-fed sham-operated (Sham) mice. OVX mice showed the increased expression of C/EBPα mRNA compared with Sham mice. However, 17β-estradiol treatment in OVX mice inhibited OVX induced-C/EBPα activation, indicating that 17β-estradiol may act as an inhibitor of C/EBPα action. Moreover, 17β-estradiol decreased mRNA levels of adipocyte marker genes, such as lipoprotein lipase, FAT/CD36 and leptin, to levels in Sham mice. These results suggest that down-regulation of adipogenesis by 17β-estradiol may be due to reduced adipose C/EBPα activities in female OVX C57BL/6 mice.

Key Words: 17β-estradiol, C/EBPα, Adipogenesis, Female mice

INTRODUCTION

Obesity is a significant risk factor for various metabolic diseases such as non-insulin-dependent diabetes, cardiovascular disease, osteoarthritis, some types of cancer, and certain reproductive and metabolic disorders (Bray, 2003). Gonadal sex steroid hormone is known to regulate obesity and lipid metabolism (Mystkwski and Schwart Z, 2000). Systemic loss of estrogen at menopause is associated with increased adiposity, which is implicated in the elevated risk of age-related metabolic diseases in women (Carr, 2003; Tchernof et al., 2004). Estrogen replacement alone, or in combination with progesterone can prevent menopause-induced gains in adipose tissue mass (Gambacciani et al., 2001; Sumino et al., 2003). To understand the pathophysiology of obesity, it is important in the study for the mechanism of adipogenesis since obesity is characterized by increased adipose tissue mass that results from both increased fat-cell number (hyperplasia) and increased fat-cell size (hypertrophy) (Couillard et al., 2000).

Adipogenesis involves dramatic changes in cell morphology and gene expression. The recent advances suggest that peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) are a key regulator of adipose cell development (Tanaka et al., 1997; Spiegelman, 1998; Tang et al., 2003). Three members of the C/EBP family, α, β, and δ, have been shown to play important roles in regulating adipose tissue development in mice and preadipocyte differentiation *in vitro* (Darlington and Ross, 1998), and be regulated by a variety of biological

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effectors (Gregoire et al., 1998; Rosen and Spiegelman, 2000). In the course of differentiation of adipocytes, PPARγ and C/EBPs cooperatively induce adipogenic genes, including adipsin, TNFα and leptin (Zhang et al., 1994; Kern et al., 1995; Alessi et al., 1997).

The phytoestrogen genistein down-regulates the expression of PPARγ and C/EBPα in 3T3-L1 cells by inhibiting C/EBPβ activity (Harmon et al., 2002). Estrogen favored early osteogenic commitment and inhibited adipogensis of mouse ST2 cells overexpressing either estrogen receptor (ER) α or β (Okazaki et al., 2002). However, the molecular and cell-biological mechanisms underlying the metabolic actions of estrogen on adipogensis are poorly understood.

We hypothesized that increased adiposity is prevented by estrogen replacement in the female ovariectomized (OVX) C57BL/6 mouse model of menopause, and these metabolic effects reflect the inhibitory action of estrogen on C/EBPα-regulated gene expression. Thus, we examined whether estrogen regulates adipose tissue mass and the expression of adipocyte-specific genes in adipose tissue of female OVX mice.

MATERIALS AND METHODS

1. Animal treatments

For all experiments, eight-week-old mice (C57BL/6) were housed and bred at the Korea Research Institute of Bioscience and Biotechnology under pathogen-free conditions with a standard 12-h light/dark cycle. Prior to the administration of special diets, mice were fed standard rodent chow and water *ad libitum*. Female mice were each randomly divided into three groups (n=5/group). The first group was sham-operated (Sham) mice. The second group was ovariectomized (OVX). The third group was OVX and subcutaneously implanted with 17β-estadiol (OVX + E, 0.05 mg/pellet, 60-day release) for 7 days. All the animals received a high fat diet (45% kcal fat, Research Diets, New Brunswick, NJ) and were sacrificed by cervical dislocation. Tissues were harvested, weighed, snap frozen in liquid nitrogen and stored at -80℃ until use.

2. RNA prepartion and analysis

Total RNA was prepared using Trizol reagent (Gibco-BRL, Grand Island, NY) and relative levels of specific mRNA were assessed by reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from RNA samples by mixing 2 μg total RNA and 0.5 μg the reverse primer in a total volume of 14 μl in water, heating the mixture at 75℃ for 15 min, cooling the mixture immediately on ice for 5 min, and adding 5X M-MLV reaction buffer, 10 mM dNTP mixture (Promega) and 200 units M-MLV RT (Promega) in total volume of 25 μl. Samples were incubated at 42℃ for 60 min. A five μl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Twenty five μl PCR sample contained 5 μl of the RT reaction, 10X buffer with $MgCl_2$, 10 mM dNTP, 5 units of Taq polymerase (Solgent, Taejon, Korea) and 10 μM of each primer. Primer sequences and PCR conditions are shown in Table 1. PCR was performed in a PTC-100™

Table 1. Sequences of oligonucleotide primers and PCR conditions

Genes	Size (bp)	Primer sequences	Annealing (℃)	Cycle
C/EBPα	465	Forward: 5'-agacatcagcgcctacatcg-3' Reverse: 5'-tgcaggtgcatggtggtc-3'	52	34
LPL	770	Forward: 5'-atggagagcaaagccctgc-3' Reverse: 5'-agtcctctctctgcaatcca-3'	52	34
FAT/CD36	883	Forward: 5'-agttttggatctttgatgtgc-3' Reverse: 5'-ttcaataggttctgaaacatc-3'	52	34
Leptin	275	Forward: 5'-ccaagaagagggatccctgctccagcagc-3' Reverse: 5'-agaatggggtgaagcccagga-3'	58	26
β-actin	350	Forward: 5'-tggaatcctgtggcatccatgaaa-3' Reverse: 5'-taaaacgcagctcagtaacagtcc-3'	58	28

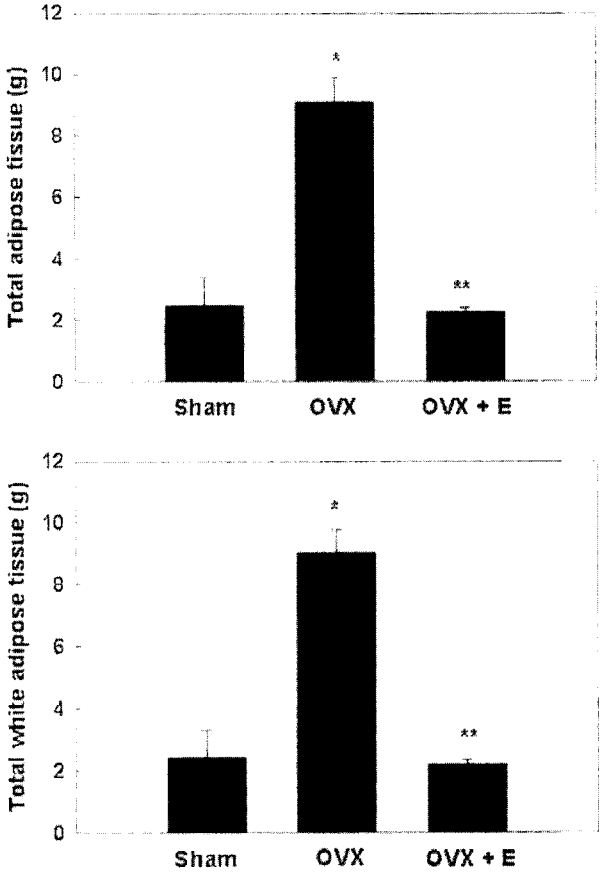

Fig. 1. Effect of 17β-estradiol on adipose tissue mass in female OVX mice. Sham-operated (Sham) and ovariectomized (OVX) mice were treated with vehicle or 17β-estradiol (OVX + E). All values are expressed as mean ± SD. *P<0.05 Significantly different from Sham mice. **P<0.05 Significantly different from vehicle-treated OVX mice.

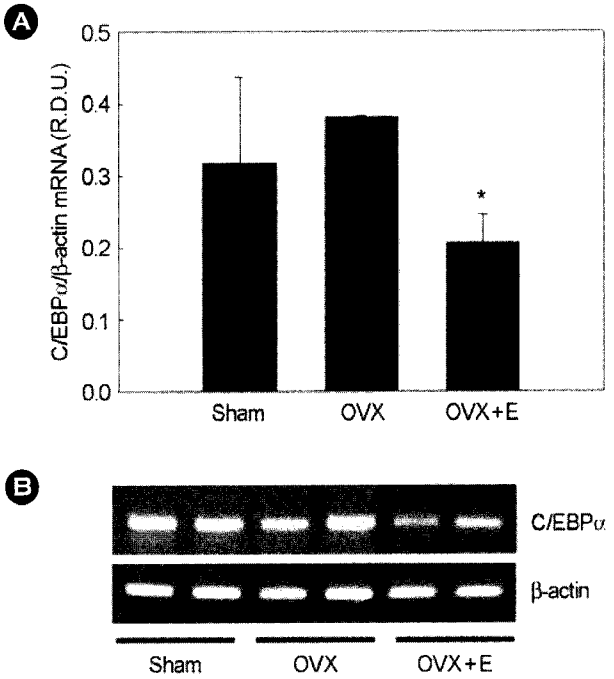

Fig. 2. Modulation of adipose C/EBPα gene expression by 17β-estradiol in female OVX mice. **(A)** Sham-operated (Sham) and ovariectomized (OVX) mice were treated with vehicle or 17β-estradiol (OVX + E). RNA was extracted from the white adipose tissue, and all values are expressed as mean ± SD of R.D.U. (relative density units) using β-actin. **(B)** Representative RT-PCR photographs from three independent experiments are shown. *P<0.05 Significantly different from vehicle-treated OVX mice.

Programmable Thermal Controller (MJ Research, Watertown, MA, USA). PCR products were electophoresed on a 1% agarose gel and quantified using the GeneGenius (Syngene, Cambridge, UK).

3. Statistics

Unless otherwise noted, all values are expressed as mean ± standard deviation (SD). All data were analyzed by ANOVA for statistically significant differences among three groups.

RESULTS

To determine whether 17β-estradiol regulates adipogenesis in female OVX C57BL/6 mice, we preferentially measured adipose tissue mass (Fig. 1). Compared with high fat diet-fed Sham mice, high fat diet-fed OVX mice significantly increased adipose tissue mass (P<0.05). In response to 17β-estradiol, an OVX-induced increase of adipose tissue was decreased (P<0.05). The total adipose tissue and white adipose tissue mass were 306.7% and 311.7% lower in 17β-estradiol-treated OVX mice, respectively, compared to OVX mice.

The expression levels of C/EBPα transcription factors were determined in adipose tissues of female mice using RT-PCR (Fig. 2). OVX mice exhibited higher mRNA expression of C/EBPα compared with Sham mice. However, these effects were inhibited by 17β-estradiol. Compared with the OVX mice, 17β-estradiol-treated OVX mice had substantially decreased levels of C/EBPα mRNA by 84%.

We next studied the modulation of adipocyte marker gene expression by 17β-estradiol in adipose tissues (Fig. 3). OVX mice had higher mRNA levels of adipocyte marker genes, such as lipoprotein lipase (LPL), fatty acid transport protein (FAT/CD36) and leptin, than Sham mice. In parallel with reductions in C/EBPα mRNA levels by 17β-estradiol, 17β-estradiol decreased mRNA expression of LPL, FAT/

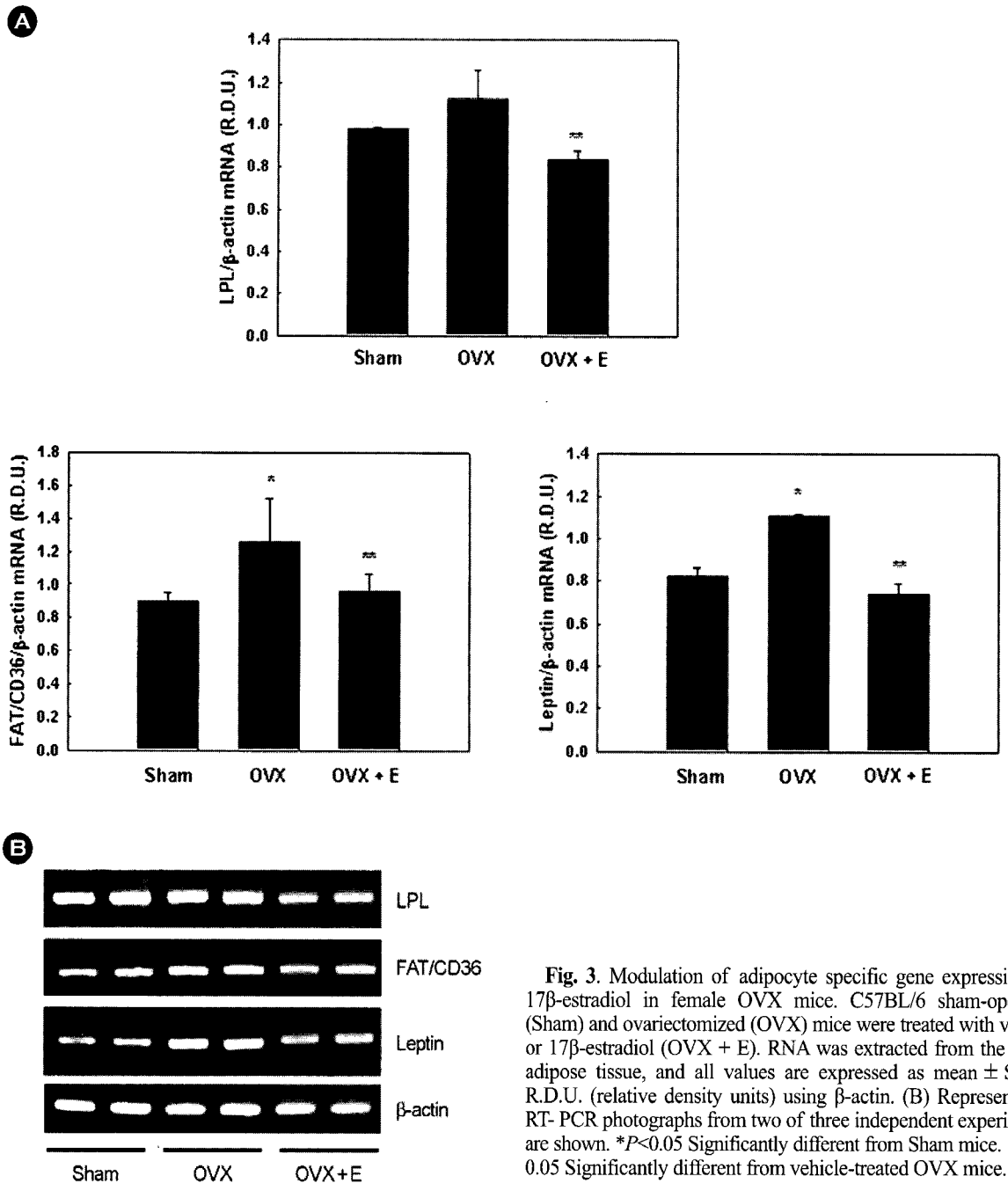

Fig. 3. Modulation of adipocyte specific gene expression by 17β-estradiol in female OVX mice. C57BL/6 sham-operated (Sham) and ovariectomized (OVX) mice were treated with vehicle or 17β-estradiol (OVX + E). RNA was extracted from the white adipose tissue, and all values are expressed as mean ± SD of R.D.U. (relative density units) using β-actin. (B) Representative RT- PCR photographs from two of three independent experiments are shown. *P<0.05 Significantly different from Sham mice. ** P< 0.05 Significantly different from vehicle-treated OVX mice.

CD36, and leptin. Since the expression of adipocyte marker genes are known to be regulated by C/EBPα activity, these results suggest that 17β-estradiol may prevent the expression of C/EBPα-mediated adipocyte marker genes, resulting in decreased adipose tissue mass and adipogenesis in female OVX mice.

DISCUSSION

In the present study, our results demonstrated that 17β-estradiol decreased adipose tissue mass and mRNA levels of adipocyte specific genes in female OVX mice, and these effects were mediated in part through the inhibition of C/EBPα activity.

The reductions in adipose tissue mass by 17β-estradiol in OVX mice are explained by the phenotype of both ERα knockout and aromatase-deficient mice. Both male and female ERα knockout mice showed significant increases in adipose tissue mass compared to wild-type mice (Heine et al., 2000), suggesting that 17β-estradiol normally has anti-

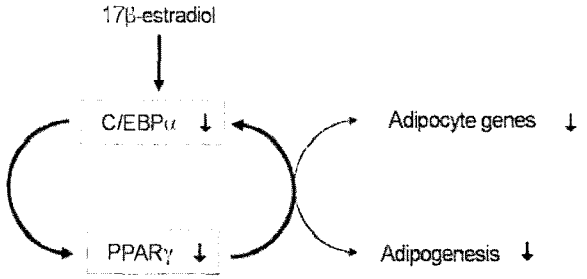

Fig. 4. The transcriptional inhibition of aipogenesis by 17β-estradiol. 17β-estradiol inhibits adipogenesis by reducing the expression of adipocyte specific genes via down-regulation of C/EBPα expression in adipose tissue of female OVX mice. PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT/enhancer-binding protein α.

lipogenic roles in males as well as females. Male and female ERα knockout mice also increased the adipocyte number in a fixed area, indicating that 17β-estradiol is an inhibitor of adult adipocytes. Aromatase-deficient mice have also shown that 17β-estradiol not only regulates adipose tissue in adult males, but also reduces the number of mature big adipocytes (Jones et al., 2000). These reports support our data showing that 17β-estradiol promotes a reduction of adipose tissue mass in female OVX mice.

It was reported that the increase in adipose tissue mass was due to the enlargement of the preexisting adipocytes with increased lipid accumulation (Ogawa et al., 2004; Villena et al., 2004; Yagi et al., 2004). Since white adipose tissue lipids are largely derived from circulating triglycerides, adipocyte size seems to be influenced by serum triglycerides (Bourgeois et al., 1983). Interestingly, estrogen-treated animal models promote lowering the serum triglyceride levels (Jeong and Yoon, 2007).

Adipogenesis involves the interaction and regulation between transcription factors, such as C/EBPs and PPARγ, which are a key regulator of adipose cell development. The synergistic activity of C/EBPα and PPARγ cooperate to promote adipocyte differentiation, including adipocyte gene expression. During adipocyte differentiation, C/EBPα was highly expressed and maintained the expression of PPARγ (Wu et al., 1999). The results presented in this study showed that ovariectomy induced the mRNA expression of C/EBPα, but 17β-estradiol inhibited ovariectomy-induced increases in C/EBPα mRNA. These results are supported by other reports that the phytoestrogen genistein decreases the expression of C/EBPα in 3T3-L1 cells and that estrogen inhibited adipogensis of mouse ST2 cells overexpressing either estrogen receptor (ER) α or ERβ (Harmon et al., 2002; Okazaki et al., 2002).

Hypertrophic adipocytes produce and secrete adipocyte-specific proteins, such as adipsin, LPL, FAT/CD36 and leptin. Similarly, OVX mice had high levels of LPL, FAT/CD36 and leptin mRNA levels compare with Sham mice. Consistent with the effects of 17β-estradiol on C/EBPα expression, administration of 17β-estradiol to OVX mice decreased the expression of LPL, FAT/CD36 and leptin genes compared with OVX mice. C/EBPα and PPARγ are suggested to bind to the promoters and activate adipocyte specific genes (Park et al., 1990; Tontonoz et al., 1994; Tontonoz et al., 1995; Hollenberg et al., 1997; Qiao et al., 2008). In the course of differentiation of adipocytes, PPARγ and C/EBPs cooperatively induce adipogenic genes, including adipsin, TNFα and leptin (Zhang et al., 1994; Kern et al., 1995; Alessi et al., 1997). Several investigations have demonstrated that activation of PPARγ in a variety of different fibroblast lines results in expression of many adipogenic genes including C/EBPα (El-Jack et al., 1999; Wu et al., 1999). Through a positive feedback loop, C/EBPα can induce PPARγ expression. In fact, recently studies have suggested that cross-regulation between C/EBPα and PPARγ is important in maintaining the differentiated state (Clarke et al., 1997; Elberg et al., 2000). C/EBPα and PPARγ induce the expression of each other and synergistically to promote adipogenesis by transactivating the promoters of several adipocyte-specific genes (Cornelius et al., 1994; Rosen et al., 2002).

In conclusion, our data provide evidence that down-regulation of adipogenesis by 17β-estradiol may be due to reductions in both C/EBPα activity and subsequent adipocyte-specific gene expression in female OVX mice (Fig. 4).

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