17β-estradiol induces an interaction between adenosine monophosphate-activated protein kinase and the insulin signaling pathway in 3T3-L1 adipocytes

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Abstract. Estrogen (17β-estradiol) has been implicated in maintaining insulin sensitivity. It is thought to act predominantly through genomic pathways and regulate the expression of various genes via binding to estrogen receptors (ERs)-α and -β. 17β-estradiol has been reported to simultaneously stimulate protein kinase B (Akt) and adenosine monophosphate-activated protein kinase (AMPK) in *ex vivo* skeletal muscle. Since data regarding the interaction between AMPK and the insulin receptor substrate-1 (IRS-1)/Akt pathway are controversial, the correlation between AMPK activation and insulin signaling remains unclear. In this study, we examined whether 17β-estradiol simultaneously stimulates the activation of AMPK and IRS-1/Akt in 3T3-L1 adipocytes as well as the 17β-estradiol-ER-induced interaction between the AMPK and IRS-1/Akt pathway in 3T3-L1 adipocytes not exposed to insulin. 17β-estradiol (10^{-7} M) rapidly activated AMPK and IRS-1/Akt in 3T3-L1 adipocytes, while the ER-α/β non-specific antagonist, ICI 182.780 (10 μM), and the AMPK antagonist compound C (20 μM) reversed the estrogen-induced activation of AMPK and tyrosine (Tyr)-IRS-1/Akt in these cells. Moreover, 17β-estradiol increased the expression of the peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC1α*), adiponectin, uncoupling protein 2 (*UCP2*) and glucose transporter 4 (*GLUT4*) genes 24 h after treatment, whereas the ER-α/β non-specific antagonist, ICI 182.780 (10 μM), and the AMPK antagonist compound C (20 μM) reversed the estrogen-induced increase in the expression of these genes. These results indicate that 17β-estradiol activates AMPK through an ER and activates Akt through AMPK activation in 3T3-L1 adipocytes, despite the absence of insulin. Furthermore, 17β-estradiol regulates the expression of genes related to glucose metabolism through ER-AMPK activation in these cells.

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Introduction

Type 2 diabetes and metabolic syndrome, both characterized by a combination of risk factors such as abdominal obesity, insulin resistance, glucose intolerance, dyslipidemia and hypertension, are more prevalent in postmenopausal women who suffer from estrogen deficiency compared to premenopausal women or men (1-3). It has been shown that long-term hormone therapy prevents diabetes and that short-term hormone therapy does not provide complete benefits in postmenopausal women (4). It has also been suggested that the effect of long-term hormone therapy in postmenopausal women may be due to altered body fat distribution and insulin sensitivity (5). Long-term estrogen treatment protects against obesity, glucose intolerance and insulin resistance in obese and insulin-resistant rodents with prolonged estrogen deficiency in the ovariectomized rodent (an animal model of menopause) (6-10). However, the molecular mechanisms underlying the short-term direct metabolic actions of estrogen on insulin sensitivity and glucose metabolism in non-obese rodents with short-term ovariectomy have not yet been clearly elucidated.

Estrogen (17β-estradiol) is considered to act predominantly through genomic pathways and regulate the expression of a number of genes via binding to estrogen receptors (ERs)-α and -β (11). Recently, the activation of non-genomic or rapid signaling pathways in response to 17β-estradiol has received increasing attention (12,13). Previous studies using ER knockout mouse models have demonstrated the role

that estrogen and its receptors play in obesity and glucose tolerance in rodents. ER-α knockout mice develop adipocyte hyperplasia and hypertrophy, insulin resistance and glucose intolerance (14). ER-α knockout male mice display an obesity phenotype after sexual maturation (15). These previous studies support the idea that 17β-estradiol plays a role in obesity, insulin sensitivity and glucose metabolism via ER-α.

Common features of the metabolic abnormalities observed in metabolic syndrome or type 2 diabetes are the dysregulation of adenosine monophosphate-activated protein kinase (AMPK) (16) and insulin resistance involving changes in the molecules of the insulin signaling cascade, such as the insulin receptor, insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) (17,18). Previous studies have shown that 17β-estradiol activates AMPK in rodent muscle (10,19,20) and C2C12 myotubes (19,21) and that 17β-estradiol upregulates PI3K/Akt signaling in human breast cancer cells through the activation of ER-α (22). It has been reported that 17β-estradiol simultaneously stimulates Akt and AMPK in *ex vivo* skeletal muscle, although 17β-estradiol does not stimulate *ex vivo* skeletal muscle glucose uptake (20). Another study has demonstrated that *in vivo* stimulation of ER-α in rats increases the activation of AMPK phosphorylation and insulin signaling proteins, such as phospho-Akt (pAkt), pAkt substrate (PAS) and insulin-stimulated skeletal muscle glucose uptake in rats (23). However, Rogers *et al* (20) suggested that 17β-estradiol does not stimulate Akt and AMPK in skeletal muscle cells, but instead stimulates these proteins in the non-skeletal muscle cells found within skeletal muscle. Since data regarding the interaction between AMPK and the IRS-1/Akt pathway are controversial, the correlation between AMPK activation and insulin signaling remains unclear.

In this study, we investigated whether 17β-estradiol simultaneously stimulates AMPK and IRS-1/Akt activation as well as the 17β-estradiol-ER-induced interaction between AMPK and the IRS-1/Akt pathway in 3T3-L1 adipocytes not exposed to insulin. Moreover, we examined whether 17β-estradiol regulates the expression of genes related to glucose metabolism through ER-AMPK activation in these cells.

Materials and methods

Materials. 17β-estradiol, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Recombinant human insulin was obtained from Eli Lilly and Company Lilly Corporate Center (Indianapolis, IN, USA). ICI 182.780 (ER-α/β non-specific antagonist) and compound C (AMPK inhibitor) were obtained from Tocris (Ellisville, MO, USA) and Calbiochem (San Diego, CA, USA), respectively. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA and penicillin/streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Anti-acetyl CoA carboxylase (ACC), anti-phospho Ser79 ACC, anti-IRS-1 and anti-phospho Ser789 IRS-1 monoclonal antibodies and anti-AMPKα and anti-phospho Thr172 AMPKα monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-phosphorylated tyrosine (pTyr)-IRS-1 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Akt and anti-phospho Ser473 Akt antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit immunoglobulin antibodies were obtained from Amersham Pharmacia Biotech, Inc. (Arlington Heights, IL, USA). The reagents for electrophoresis were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All other reagents were of analytical grade or complied with the standards required for cell culture experiments.

Cell culture and differentiation. 3T3-L1 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Two days after confluence, 3T3-L1 fibroblasts were used for differentiation (day 0). The differentiation medium, phenol red-free DMEM, contained 10% charcoal-stripped FBS (CS-FBS), 0.5 mM IBMX, 1 μM dexamethasone and 5 μg/ml insulin. After day 3, the differentiation medium was replaced with a post-differentiation medium containing 10% CS-FBS and 5 μg/ml insulin. After day 5, the post-differentiation medium was replaced with phenol red-free DMEM supplemented with 10% CS-FBS. The medium was changed every 3 days until the cells were used for experiments. Eight days after the induction of differentiation, >80-90% of the cells featured the morphological and biochemical properties of adipocytes. Before their use in experiments, the cells were starved for 12 h in phenol red-free DMEM containing 1% CS-FBS and then incubated with 10 μM ICI 182.780, 20 μM compound C, or 10^{-7} M 17β-estradiol alone or in combinations. ICI 182.780 and compound C were added 30 min prior to 17β-estradiol treatment for the indicated time and dose.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the 3T3-L1 adipocytes using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Equal amounts of total RNA were reverse-transcribed into cDNA using the ImProm-II™ reverse transcriptase system (Promega Corp., Madison, WI, USA) and an oligo$(dT)_{15}$ primer (Promega Corp.). The peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC1α*), adiponectin, uncoupling protein 2 (*UCP2*) and glucose transporter 4 (*GLUT4*) genes were selected for the experiments as the genes related to glucose metabolism. The primers used were as follows: *PGC1α*, 5'-TATGGTTTCATCACCTACCG-3' and 5'-CGTCCACAAAAGTACAGCTC-3'; adiponectin, 5'-CCGTTCTCTTCACCTACGACC-3' and 5'-GGGCTATGGGTAGTTGCAGTC-3'; *UCP2*, 5'-AGATACATGAACTCTGCCTTGGG-3' and 5'-GGCAGAGGATGAAGAAAAAGAC-3'; *GLUT4*, 5'-GTGTGTGAGCGAGTGCTTTCC-3' and 5'-TGGAGACTGATGCGCTCTAACC-3' and β-actin, 5'-ACGGCCAGGTCATCACTATT-3' and 5'-AATGTAGTTTCATGGATGCC-3'. RT-PCR was conducted using the DNA Engine Opticon System (MJ Research, Inc., South San Francisco, CA, USA) in a 20-μl reaction mixture containing 10 μl of SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan), 10 pmol of forward primer, 10 pmol of reverse primer and 1 μg of cDNA. Amplification parameters consisted of an initial denaturation at 95˚C for 5 min and 40 cycles of 3-step PCR (denaturation at 95˚C for 1 min, annealing at 52-57˚C for

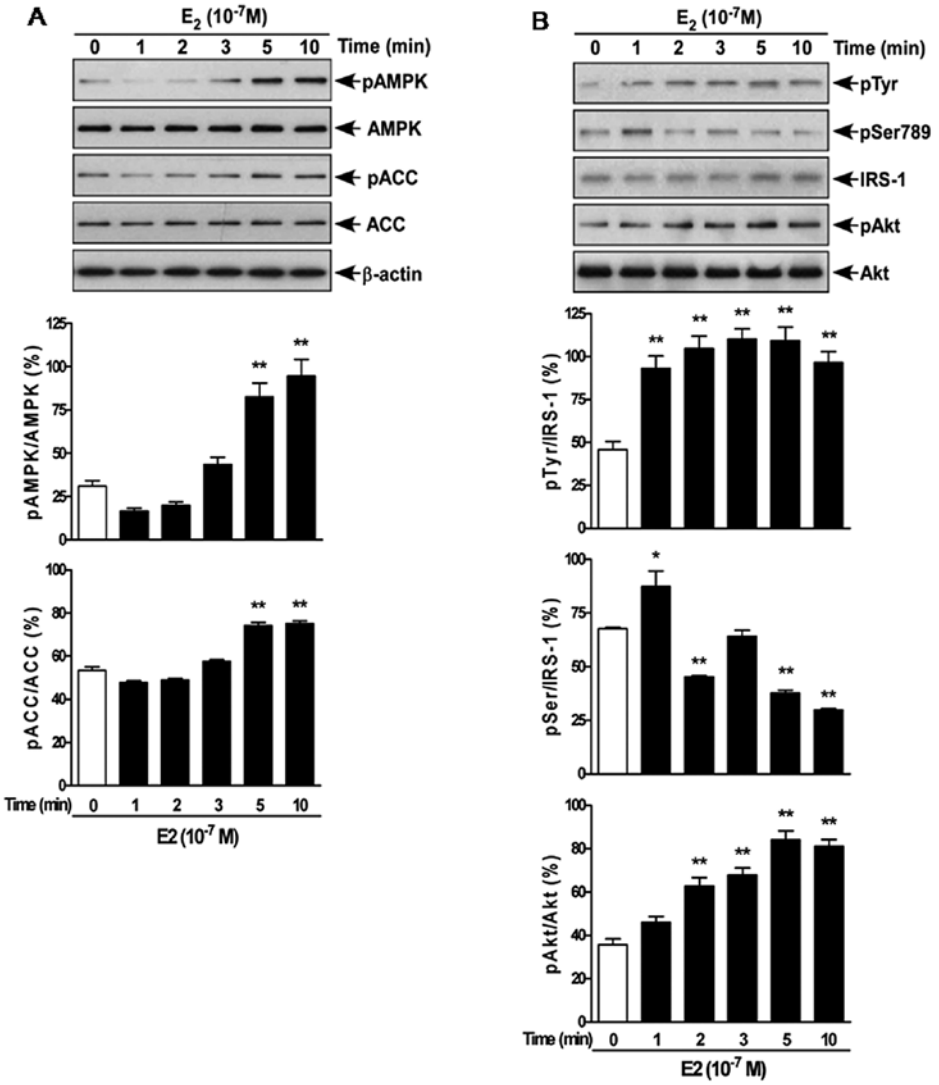

Figure 1. Estrogen stimulates AMPK and the insulin signaling pathway in 3T3-L1 adipocytes. (A) Representative western blot analyses of total AMPK, phospho-AMPK (pAMPK), total ACC and phospho-ACC (pACC) (upper panel); the phospho-AMPK to AMPK protein ratio and the phospho-ACC to ACC protein ratio (lower panel). (B) Representative western blot analyses of the total IRS-1, phospho-Tyr-IRS-1, phospho-Ser789-IRS-1, total Akt and phospho-Ser473-Akt (upper panel); and the phospho-Tyr-IRS-1 to IRS-1, phospho-Ser789-IRS-1 to IRS-1 and phospho-Ser473-Akt to Akt protein ratios (lower panel). The phospho-protein to total protein ratios are presented as the means ± SD and are represented as the percentage of expression. *P<0.05, **P<0.01 vs. the expression at zero time.

30 sec and extension at 72˚C for 1 min). Data were analyzed using a comparative critical threshold (Ct) method with the amount of the target gene normalized to the average of the control gene (β-actin). The percentage difference was calculated by $2^{-\Delta\Delta Ct}$. Each experiment was performed at least 4 times.

Western blot analysis. Whole cell lysates were prepared by lysing cells in Pro-prep protein extraction solution (Intron Biotechnology, Seoul, Korea) containing 10 mM sodium phosphate (pH 7), 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 4 μg/ml leupeptin and 1 mM PMSF. The protein concentration of the lysates was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc.). Equal amounts of protein (20 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4-20% gel) and transferred by electroblotting onto nitrocellulose membranes (Invitrogen Life Technologies). The membranes were then blocked with 5% non-fat dry milk and probed with primary antibodies. After being washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit immunoglobulin antibodies (1:500) (Amersham Pharmacia Biotech, Inc.). After washing with PBS containing 0.1% Tween-20, the specific signals were detected with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Inc.). The band densities were quantified using ImageJ software (http://rsb.info.nih.gov/ij/). Each experiment was performed at least 3 times.

Statistical analyses. Data are presented as the means ± standard deviation (SD). All statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS; Korean version 20.0). The statistical differences were analyzed using one-way analysis of variance (ANOVA)

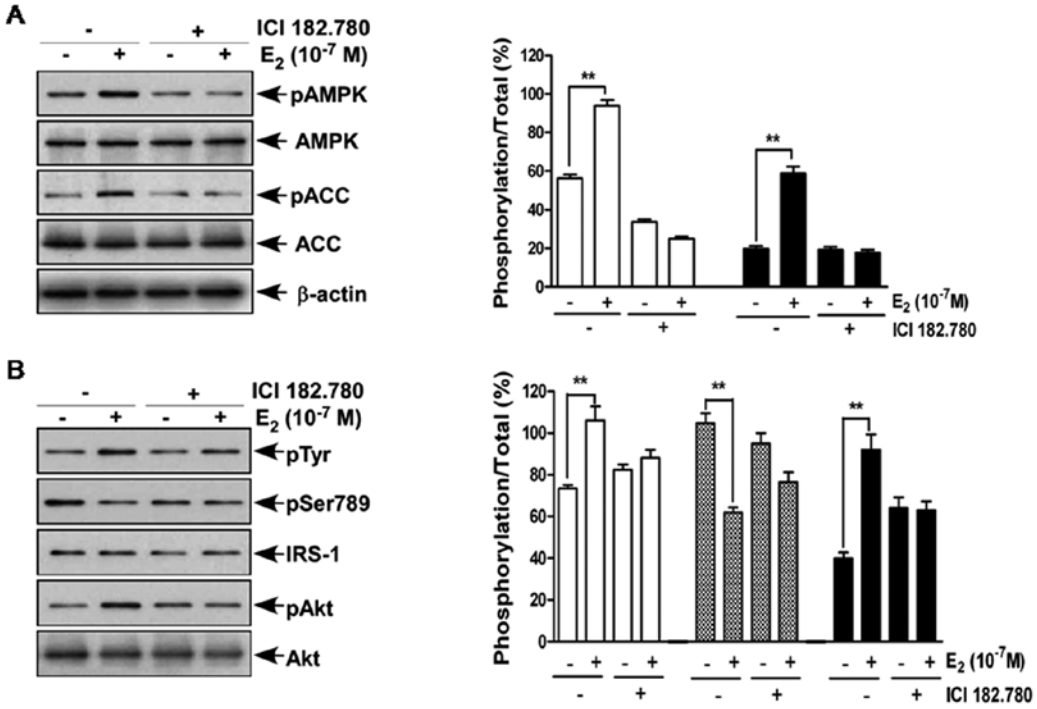

Figure 2. The estrogen receptor antagonist, ICI 182.780, attenuates estrogen-induced signaling in 3T3-L1 adipocytes. Representative western blot analyses of the molecular changes of AMPK (A) and insulin signaling (B) (left panel) and the phospho-protein to total protein ratios (right panel) in 3T3-L1 adipocytes are shown (white columns, phospho-AMPK to AMPK ratio or phospho-Tyr-IRS-1 to IRS-1 ratio; grey columns, phospho-Ser789 to IRS-1 ratio; black columns, phospho-ACC to ACC ratio or phospho-Ser473-Akt to Akt ratio). The phospho-protein to total protein ratios are presented as the means ± SD and are represented as the percentage of expression. **P<0.01.

followed by Tukey's test. Statistical significance was defined as P<0.05.

Results

17β-estradiol rapidly activates AMPK and IRS-1/Akt in 3T3-L1 adipocytes. 17β-estradiol (10^{-7} M) phosphorylated AMPK and its substrate, ACC, within 10 min in 3T3-L1 adipocytes which were not exposed to insulin (Fig. 1A). 17β-estradiol (10^{-7} M) also pTyr-IRS-1 and Akt and decreased the phosphorylation of Ser789-IRS-1, whose level increases with insulin resistance (18), within 10 min in 3T3-L1 adipocytes not exposed to insulin (Fig. 1B). These results indicate that 17β-estradiol rapidly activates AMPK and IRS-1/Akt in 3T3-L1 adipocytes without insulin-induced signaling activation.

17β-estradiol activates AMPK and IRS-1/Akt through an ER in 3T3-L1 adipocytes. 17β-estradiol (10^{-7} M) phosphorylated AMPK, its substrate ACC, Tyr-IRS-1 and Akt and decreased the phosphorylation of Ser789-IRS-1 10 min after treatment in 3T3-L1 adipocytes not exposed to insulin (Fig. 2). However, the ER-α/β non-specific antagonist, ICI 182.780 (10 μM), reversed the estrogen-induced activation of AMPK, ACC and Tyr-IRS-1/Akt and the estrogen-induced decrease in Ser789-IRS-1 phosphorylation in 3T3-L1 adipocytes not exposed to insulin (Fig. 2). These results indicate that 17β-estradiol activates AMPK and IRS-1/Akt through an ER in 3T3-L1 adipocytes without insulin-induced signaling activation.

17β-estradiol activates IRS-1/Akt through AMPK activation in 3T3-L1 adipocytes. 17β-estradiol (10^{-7} M) phosphorylated AMPK, its substrate ACC, Tyr-IRS-1 and Akt and decreased Ser789-IRS-1 phosphorylation 10 min after treatment in 3T3-L1 adipocytes not exposed to insulin (Fig. 3). By contrast, the AMPK antagonist compound C (20 μM) reversed the estrogen-induced activation of AMPK, ACC and Tyr-IRS-1/Akt and the estrogen-induced decrease in Ser789-IRS-1 phosphorylation in 3T3-L1 adipocytes not exposed to insulin (Fig. 3). These results indicate that 17β-estradiol activates IRS-1/Akt through AMPK activation in 3T3-L1 adipocytes without insulin-induced signaling activation.

17β-estradiol regulates genes related to glucose metabolism through ER-AMPK activation in 3T3-L1 adipocytes. 17β-estradiol (10^{-7} M) increased the expression of *PGC1α*, the adiponectin gene, *UCP2* and *GLUT4* at 24 h after treatment in 3T3-L1 adipocytes not exposed to insulin (Fig. 4). However, the ER-α/β non-specific antagonist, ICI 182.780 (10 μM), reversed the estrogen-induced increase in *PGC1α*, the adiponectin gene, *UCP2* and *GLUT4* expression 24 h after 17β-estradiol (10^{-7} M) treatment in 3T3-L1 adipocytes not exposed to insulin. In addition, the AMPK antagonist compound C (20 μM) also reversed the estrogen-induced increase in *PGC1α*, the adiponectin gene, *UCP2* and *GLUT4* expression 24 h after 17β-estradiol (10^{-7} M) treatment in 3T3-L1 adipocytes not exposed to insulin. These results indicate that 17β-estradiol regulates genes related to glucose metabolism through ER-AMPK activation in 3T3-L1 adipocytes without insulin-induced signaling activation.

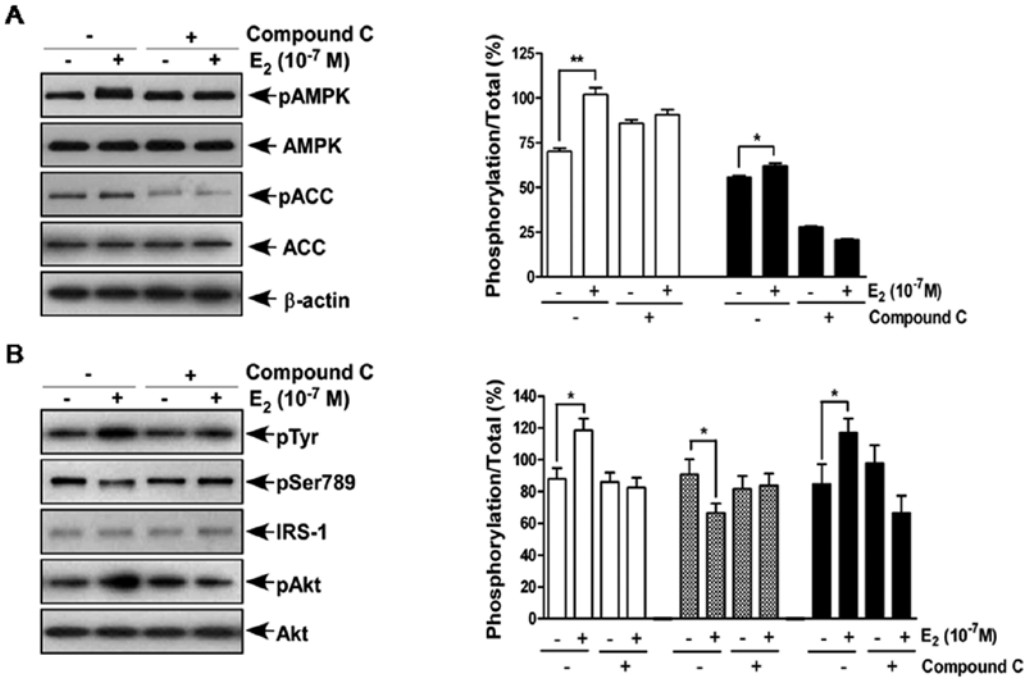

Figure 3. The AMPK inhibitor compound C attenuates estrogen-induced signaling in 3T3-L1 adipocytes. Representative western blot analyses of the molecular changes of AMPK (A) and insulin signaling (B) (left panel) and the phospho-protein to total protein ratios (right panel) in 3T3-L1 adipocytes are shown (white columns, phospho-AMPK to AMPK ratio or phospho-Tyr-IRS-1 to IRS-1 ratio; grey columns, phospho-Ser789 to IRS-1 ratio; black columns, phospho-ACC to ACC ratio or phospho-Ser473-Akt to Akt ratio). The phospho-protein to total protein ratios are presented as the means ± SD and are represented as the percentage of expression. *P<0.05, **P<0.01.

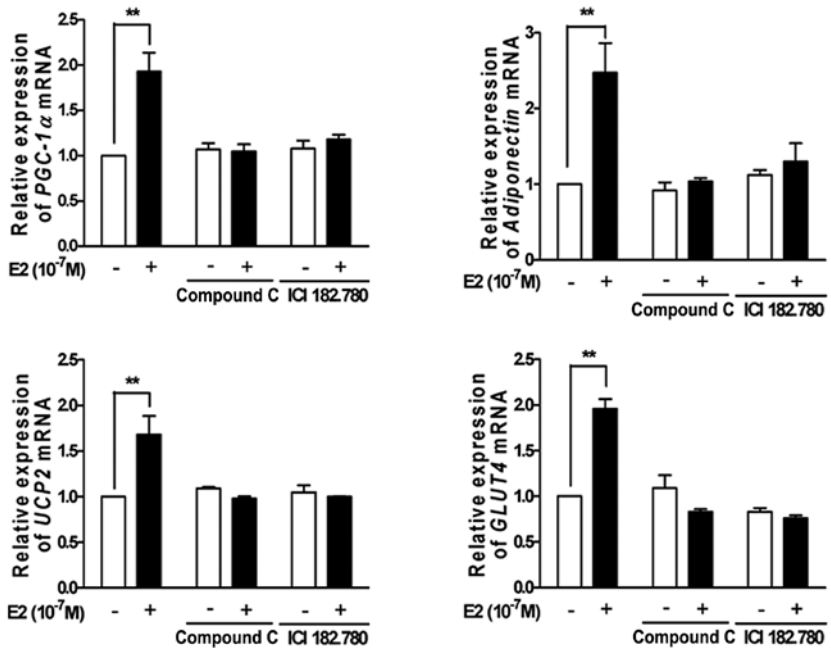

Figure 4. Estrogen alters the expression of genes related to glucose and lipid metabolism in 3T3-L1 adipocytes. The expression levels of *PGC1α*, the adiponectin gene, the resistin gene, *UCP2* and *GLUT4* are shown. The estrogen receptor antagonist (ICI 182.780) and AMPK inhibitor (compound C) attenuated estrogen-induced signaling in 3T3-L1 adipocytes. The mRNA expression levels are presented as the threshold cycle (Ct) value normalized to the β-actin mRNA value. **P<0.01.

Discussion

In the present study, we demonstrate that 17β-estradiol simultaneously stimulates the activation of IRS-1/Akt and AMPK and induces an interaction between AMPK and the IRS-1/Akt signaling pathway via an ER in 3T3-L1 adipocytes, despite the absence of insulin. Our data also demonstrate that an estrogen/ER-induced activation of AMPK functions upstream

of IRS-1/Akt signaling and estrogen regulates the expression of genes related to glucose metabolism through 17β-estradiol-ER-AMPK activation in 3T3-L1 adipocytes in the absence of insulin-induced signaling activation.

Given that data concerning the simultaneous stimulation of IRS-1/Akt and AMPK signaling and the interaction between AMPK and IRS-1/Akt pathway remain controversial, this type of interaction may be highly cell type-dependent. The present study demonstrates that 17β-estradiol simultaneously stimulates the activation of IRS-1/Akt and AMPK and induces an interaction between AMPK and the IRS-1/Akt pathway without insulin-induced signaling activation in 3T3-L1 adipocytes. In addition, we show that AMPK functions upstream of Akt signaling in 3T3-L1 adipocytes, consistent with a previous study using human umbilical vein endothelium cells (HUVECs) (24). However, this result is in direct contrast with the results of another study showing that AMPK functions downstream of the PI3K pathway in vascular endothelial cells (25). Further research in various cell types or tissues is required to clarify this phenomenon.

Although we show that estrogen simultaneously activates AMPK and IRS-1/Akt via ER 10 min after treatment, the mechanism involved remains unclear. In a previous study, ER-α was shown to regulate insulin sensitivity through IRS-1 tyrosine phosphorylation in mature 3T3-L1 adipocytes (26). Additionally, a previous study demonstrated that ER-α regulates insulin sensitivity and glucose metabolism (15,27). On the basis of these observations, we postulated that 17β-estradiol may activate AMPK and IRS-1/Akt through ER-α. Further research on the activation of AMPK and IRS-1/Akt through the G protein-coupled receptor 30 (GPR30) or G protein-coupled ER (GPER) by 17β-estradiol is required as these receptors have recently been implicated in mediating several of the non-genomic or rapid signaling effects of 17β-estradiol (13,28,29). In the present study, 17β-estradiol (10^{-7} M) increased the expression of the glucose metabolism-related genes, *PGC1α*, the adiponectin gene, *UCP2* and *GLUT4*, 24 h after treatment through 17β-estradiol-ER-AMPK activation in 3T3-L1 adipocytes without insulin-induced signaling activation. The co-activator protein, PGC1α, has been implicated in mitochondrial metabolism and biogenesis, thermogenesis, adipocyte differentiation and glucose metabolism, including gluconeogenesis and glucose uptake (30,31). Mitochondrial UCP2 has been implicated in physiological and pathological processes related to glucose and lipid metabolism (32). UCP2 is an inner mitochondrial membrane protein expressed in white adipose tissue and skeletal muscle (33) and is involved in energy expenditure (34). In skeletal muscle and adipose tissue, glucose uptake is maintained by one of the isoforms of the GLUT family (GLUT4) (35) and insulin resistance is associated with decreased glucose uptake in insulin-sensitive tissues, such as skeletal muscle and adipose tissue (36). The adipocytokine, adiponectin, reverses insulin resistance associated with both lipoatrophy and obesity (37). On the basis of these findings, *PGC1α*, *UCP2*, *GLUT4* and the adiponectin gene were selected as the glucose metabolism-related genes for the present study. We demonstrated that 17β-estradiol increased the expression of *PGC1α*, *UCP2*, *GLUT4* and the adiponectin gene in 3T3-L1 adipocytes, suggesting that estrogen possibly regulates insulin sensitivity, glucose metabolism and energy expenditure through the regulation of the expression levels of these genes. By contrast, an ER-α/β non-specific antagonist and an AMPK antagonist reversed the estrogen-induced increase of *PGC1α*, the adiponectin gene, *UCP2* and *GLUT4* expression, indicating that 17β-estradiol regulates the expression levels of genes related to glucose metabolism through 17β-estradiol-ER induced AMPK activation in 3T3-L1 adipocytes. Further studies are required in order to determine whether this phenomenon occurs in other cell types or tissues.

In conclusion, 17β-estradiol activates AMPK through an ER (possibly ER-α) and then activates Akt through AMPK activation in 3T3-L1 adipocytes, despite the absence of insulin. Moreover, 17β-estradiol also regulates the expression levels of genes related to glucose metabolism through ER-AMPK activation in 3T3-L1 adipocytes, despite the absence of insulin. Further research is required to elucidate the precise mechanisms underlying the estrogen-induced interaction between AMPK and the IRS-1/Akt pathway and the regulation of gene expression related to glucose metabolism.

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