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Estradiol Stimulates Akt, AMPK and TBC1D1/4, But Not Glucose Uptake in Rat Soleus*

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Abstract

Post-menopausal women exhibit decreases in circulating estrogen levels and whole body insulin sensitivity, suggesting that estrogen regulates skeletal muscle glucose disposal. Thus, we assessed whether estrogen stimulates glucose uptake or enhances insulin sensitivity in skeletal muscle. *Ex vivo* muscle stimulation with 17 β -estradiol (10 nM) resulted in a rapid (≤ 10 min) increase in the phosphorylation of Akt, AMP-activated protein kinase (AMPK), and TBC1D1/4, key signaling proteins that regulate glucose uptake in muscle. Treatment with the estrogen receptor antagonist, ICI 182,780, only partly inhibited signaling, suggesting both an estrogen receptor-dependent and independent mechanism of estradiol action. 17 β -Estradiol did not stimulate *ex vivo* muscle [³H]-2-deoxyglucose uptake or enhance insulin-induced glucose uptake, demonstrating discordance between the estradiol-induced stimulation of signaling proteins and muscle glucose uptake. This study is the first to demonstrate that estradiol stimulates Akt, AMPK, and TBC1D1/4 in intact skeletal muscle, but surprisingly, estradiol does not stimulate muscle glucose uptake.

Keywords

estrogen; ICI 182; 780; skeletal muscle; AS160; insulin signaling

INTRODUCTION

Menopause is a non-pathological, age-related decline in female estrogen production. After menopause women are at a greater risk for developing type 2 diabetes [1], a metabolic disorder defined by an inability of insulin-sensitive target tissues (e.g. skeletal muscle, adipose tissue, etc) to respond properly to insulin. Estrogen replacement therapy can ameliorate the menopause-induced increase in type 2 diabetes risk [2], suggesting a critical role for estrogen in regulating whole body glucose metabolism. As women can now expect to live at least a third of their lives in a post-menopausal state [3], understanding the role that estrogen plays in regulating glucose metabolism may provide key insights towards reducing the incidence of type 2 diabetes worldwide.

* AMPK = AMP-activated protein kinase

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Skeletal muscle is the major tissue responsible for uptake of glucose from the blood, accounting for 70–85% of whole body glucose disposal [4]. There are two widely studied physiological stimuli that increase muscle glucose uptake, insulin and exercise/muscle contraction, and both of these stimuli increase glucose uptake via the activation of intracellular signaling cascades (reviewed in [5]). The signaling mechanism by which insulin stimulates muscle glucose uptake is relatively well known and involves binding of insulin to the insulin receptor, phosphorylation of the serine/threonine kinase, Akt, and phosphorylation of the Rab-GTPase activating protein, TBC1D4 [also known as Akt substrate of 160 kDa (AS160)] (reviewed in [6] and [7]). In contrast, the signaling mechanism(s) by which exercise acts is still relatively unknown, although studies have shown that activation of the energy sensing kinase, AMP-activated protein kinase (AMPK), has been positively correlated with increases in muscle glucose uptake [8]. In addition, recent work has shown that phosphorylation of TBC1D4 can also be regulated by exercise/muscle contraction and AMPK activation in muscle [9], suggesting that phosphorylation of TBC1D4 may be a critical step linking both Akt-dependent and AMPK-dependent signals to the regulation of muscle glucose uptake (reviewed in [7]).

Intriguingly, recent work has shown that acute stimulation of C2C12 skeletal muscle cells with physiological doses of the estrogen, 17 β -estradiol, increased the phosphorylation of Akt [10] and AMPK [11;12], suggesting that the beneficial effect of estrogen replacement therapy on type 2 diabetes incidence in post-menopausal women may be to stimulate skeletal muscle glucose uptake. However, it is presently unclear whether estrogen can stimulate Akt, AMPK or TBC1D1/4 in intact skeletal muscle, or whether the activation of these signaling proteins by estradiol increases muscle glucose uptake. Thus, the aim of the current study was to determine whether 17 β -estradiol stimulates key intracellular signals known to regulate glucose metabolism (Akt, AMPK, and TBC1D1/4) and/or glucose uptake in intact skeletal muscle.

MATERIALS AND METHODS

Animals

Experiments were performed in accordance with the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and National Institutes of Health guidelines for the care and use of laboratory animals. Female Sprague-Dawley rats (40–50 g) from Taconic Labs (Germantown, NY) were housed at constant temperature (20–22°C) with a 12 hr light/dark cycle. LabDiet® chow (Purina Mills Inc, St. Louis, MO) and water were available ad libitum.

Ex vivo Skeletal Muscle Incubations

Skeletal muscle incubation experiments were performed as previously described [9]. Briefly, rats were fasted 4 hrs and sacrificed by cervical dislocation. Soleus muscles were excised and incubated in continuously gassed (95% O₂/5% CO₂) Krebs–Ringer Bicarbonate (KRB) buffer containing (in mM): 117 NaCl, 4.7 KCl, 2.5 CaCl₂•2H₂O, 1.2 KH₂PO₄, 1.2 MgSO₄•7H₂O, 24.6 NaHCO₃, pH 7.5, plus 5.6 mM glucose. For signaling studies, muscles were pre-incubated in KRB buffer containing the estrogen receptor antagonist, ICI 182,780 (Tocris, Ellisville, MO) or DMSO (0.1%) for 30 min prior to any additional perturbation. 17 β -estradiol (10 nM; Sigma, St Louis, MO) was dissolved in 100% ethanol and added 1, 2, 5 or 10 min prior to the end of the experiment. Muscles were frozen in liquid N₂.

Ex vivo Skeletal Muscle [³H]-2-Deoxyglucose Uptake

Muscle incubations were performed as described above except that KRB buffer was supplemented with 2 mM pyruvate instead of glucose. Muscles were stimulated with 17 β -estradiol (10 nM) for 10 min, and then transferred to tubes containing KRB buffer supplemented with 1.5 μ Ci/ml [³H]-2-deoxyglucose, 1 mM deoxyglucose, 0.45 μ Ci/ml [¹⁴C]-mannitol, 7 mM cold-mannitol and the appropriate amount of ethanol, 17 β -estradiol, and/or

insulin. After 10 min, muscles were frozen in liquid N₂ to terminate glucose uptake. Frozen muscles were solubilized in 1 M NaOH at 80°C and neutralized with 1 M HCl. Non-soluble particulates were precipitated by centrifugation at 13,000 × g for 1 min. Radioactivity in samples was assessed by liquid scintillation counting for [³H] and [¹⁴C], and extracellular and intracellular spaces calculated to determine glucose uptake.

Immunoblotting

Immunoblot analyses were performed using standard procedures. Briefly, frozen muscles were homogenized on ice in lysis buffer containing (in mM): 20 Tris-HCl (pH 7.4), 50 NaCl, 50 NaF, 5 Na₄P₂O₇, 250 sucrose, 1% Triton-X, 2 dithiothreitol, 2.5 soybean trypsin inhibitor, 0.008 leupeptin, 0.1 benzamidine, 1 4-(2-aminoethyl)benzenesulfonylfluoride, 1 Na₃VO₄, and 0.002 aprotinin, and then centrifuged at 13,000 × g for 30 min. Total protein content was assessed via the Bradford method. Muscle lysates (25 µg) were resolved by SDS-PAGE on 4–15% gradient gels (Bio-Rad, Foster City, CA) and proteins transferred to nitrocellulose membranes. After blocking in 5% non-fat dry milk, primary antibodies (Cell Signaling Inc., Danvers, MA) were incubated with membranes overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) was incubated with membranes, and detected using enhanced chemiluminescence detection reagents (GE healthcare, Piscataway, NJ). Band density was quantified using densitometry (FluorChem 2.0; Alpha Innotech, San Leandro, CA) in the linear range of detection.

Statistics

Data are presented as the mean and standard error of the mean. Statistical significance was defined as P≤0.05 and determined by One-way or Two-way ANOVA and Tukey's post-hoc analysis.

RESULTS AND DISCUSSION

Acute effects of 17β-estradiol on skeletal muscle signaling proteins

Previous studies in C2C12 muscle cells have shown that acute treatment with 17β-estradiol rapidly increases phosphorylation of Akt [10] and AMPK [11;12], two signaling proteins implicated in the regulation of skeletal muscle glucose uptake. Thus, the first aim of this study was to determine whether estradiol elicited similar activation of these signaling proteins in intact skeletal muscle. As shown in FIGS 1A+B, treatment of rat soleus muscles with 10 nM 17β-estradiol significantly increased the phosphorylation of Akt (Ser473) and AMPK (Thr172) after 5 and 10 min, demonstrating rapid activation of these signaling proteins in skeletal muscle.

TBC1D1 and TBC1D4 are protein homologues that have recently been implicated in the regulation of muscle glucose uptake (reviewed in [7]). Interestingly, both of these proteins can be phosphorylated by both Akt and AMPK on phospho-Akt-substrate (PAS) motif sites [9; 13], key residues that mediate the ability of TBC1D1/4 to regulate glucose uptake (reviewed in [7]). Thus, we next wanted to determine if estradiol stimulates TBC1D1/4 (PAS) phosphorylation in rat skeletal muscle. As shown in FIG 1C, TBC1D1/4 (PAS) phosphorylation was increased after 5 and 10 min of estradiol treatment demonstrating for the first time, in any cell or tissue, that TBC1D1/4 (PAS) phosphorylation can be regulated by 17β-estradiol. Of note, the PAS antibody does not distinguish between TBC1D1 and TBC1D4 phosphorylation. Thus, the relative contribution of these two proteins to the PAS signal cannot be discerned using this antibody. However, since soleus muscle contains much more TBC1D4 than TBC1D1 protein [13], it is very likely that the PAS signal from the soleus muscles is mostly TBC1D4 phosphorylation.

Effects of estradiol on skeletal muscle signaling are partially mediated via estrogen receptors

There are two well-characterized estrogen receptors in mammalian cells, estrogen receptor α and estrogen receptor β , both of which are present in rat skeletal muscle [14]. Although estrogen receptors are classically known as nuclear steroid hormone receptors (reviewed in [15]), recent work has indicated that sub-populations of estrogen receptors reside in the plasma membrane and can stimulate rapid, non-genomic signaling events in response to estradiol (reviewed in [16]). Thus, we next assessed if 17β -estradiol stimulated Akt, AMPK and TBC1D1/4 via estrogen receptor α/β in rat soleus.

As shown in FIG 2B, pre-treatment of muscles with the estrogen receptor antagonist, ICI 182,780, significantly reduced the estradiol-induced increase in AMPK (Thr172) phosphorylation, suggesting that AMPK stimulation by estradiol in rat soleus is largely mediated via estrogen receptors. ICI 182,780 did not significantly inhibit the phosphorylation of Akt (Ser473), and demonstrated a trend to reducing TBC1D1/4 (PAS, $P=0.1$) (FIGS 2A+C), suggesting that stimulation of Akt by estradiol is largely mediated via an estrogen receptor-independent mechanism. One possibility for this mechanism is the plasma membrane G-protein coupled membrane receptor 30 (GPR30), which can mediate some of the signaling effects of estrogens in mammalian cells [17]. However, although GPR30 expression has been detected in human skeletal muscle [18], to date the expression of GPR30 in rodent skeletal muscle is not known. Thus, more studies need to be done to elucidate the complex mechanism by which estrogens mediate all of their rapid, non-genomic actions.

Effects of acute estradiol treatment on skeletal muscle glucose uptake and insulin sensitivity

In skeletal muscle, phosphorylation of Akt [6], AMPK [8] and TBC1D4 [19] have been positively correlated with increases in glucose uptake. Since estradiol stimulated the phosphorylation of all of these signaling proteins in rat soleus muscle, the next aim was to determine if estradiol also stimulated glucose uptake into muscle. As shown in FIG 3 (basal), despite the estradiol-induced increase in Akt (Ser473), AMPK (Thr172) and TBC1D1/4 (PAS) phosphorylation, estradiol did not increase muscle glucose uptake.

Skeletal muscle insulin sensitivity is enhanced by activation of AMPK [20]. Thus, it is possible that estrogen stimulation does not promote muscle glucose uptake, but that the activation of AMPK promotes muscle sensitivity to insulin. To assess this possibility, soleus muscles were stimulated with 17β -estradiol in the presence or absence of submaximal (150 $\mu\text{U/ml}$ – 1,200 $\mu\text{U/ml}$) or maximal (50,000 $\mu\text{U/ml}$) levels of insulin. As shown in FIG 3, estradiol did not enhance the insulin-induced stimulation of muscle glucose uptake at either submaximal or maximal doses. Collectively, these results demonstrate that acute estradiol treatment does not stimulate glucose uptake or enhance insulin sensitivity in skeletal muscle.

One possible explanation for these unusual findings is that estradiol was not actually stimulating the phosphorylation of Akt, AMPK, and TBC1D1/4 in the skeletal muscle cells, but was instead stimulating these signaling proteins in the non-muscle cells found within intact skeletal muscle, such as endothelial cells, nerve cells, etc. In fact, studies have shown that estradiol can stimulate AMPK phosphorylation in cultured endothelial cells [21]. However, this scenario is unlikely given that less than 1% of an intact skeletal muscle is comprised of endothelial cells [22], and the rapid nature of the activation would negate any possibility that estradiol was increasing protein expression of these signaling proteins. Thus, an explanation for the observed discordance is presently unclear.

The findings from this study significantly contribute to our overall understanding of the role that estrogens play in regulating whole body glucose metabolism and protecting women from

type 2 diabetes. As skeletal muscle is the primary site for blood glucose disposal, contains estrogen receptors, and is a target tissue for type 2 diabetes treatments, it was critical to determine the effects of estradiol treatment on muscle glucose uptake. Contrary to our hypothesis that estradiol may enhance whole body insulin sensitivity via stimulation of muscle intracellular signaling proteins (Akt, AMPK and TBC1D1/4) and glucose uptake, our study is the first to demonstrate that in isolated rodent skeletal muscle, activation of relevant signaling pathways by estradiol did not produce a measurable increase in glucose uptake. Importantly, these studies raise a critical question about the relative importance of the estrogen-induced activation of kinase cascades and its overall effect(s) on skeletal muscle physiology.

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FIG 1. 17 β -Estradiol stimulated phosphorylation of key signaling molecules that regulate glucose uptake in rat soleus

In isolated skeletal muscle, estradiol stimulated the phosphorylation of (A) Akt (Ser473), (B) AMP-activated protein kinase (AMPK; Thr172), and (C) TBC1D1/4 [phospho-Akt-substrate (PAS) motif sites], compared to vehicle (EtOH)-treated control muscles. (N = 8–16 muscles/group.) *P<0.05, **P<0.01, ***P<0.001 vs Vehicle.

FIG 2. The estrogen receptor antagonist, ICI 182,780, attenuated estradiol-induced signaling in rat soleus

ICI 182,780 (100 nM) pre-treatment attenuated estradiol-induced increases in (A) Akt (Ser473), (B) AMP-activated protein kinase (AMPK; Thr172) phosphorylation, and (C) TBC1D1/4 [phospho-Akt-substrate (PAS) motif site] phosphorylation. (N = 5–18 muscles/group *P<0.05, **P<0.01 vs Basal, \$P<0.05 vs Vehicle (EtOH)).

FIG 3. 17β -Estradiol does not stimulate glucose uptake or enhance insulin sensitivity in rat soleus
 Treatment of isolated skeletal muscles with estradiol (10 nM) did not increase glucose uptake (*Basal*), or enhance the ability of insulin to increase glucose uptake. (N = 4–9 muscles/group.)
 *P<0.05, **P<0.01, ***P<0.001 vs Vehicle.