Estradiol-induced regulation of GLUT4 in 3T3-L1 cells: involvement of ESR1 and AKT activation

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Abstract

Impaired insulin-stimulated glucose uptake involves reduced expression of the GLUT4 (solute carrier family 2 facilitated glucose transporter member 4, SLC2A4 gene). 17β-estradiol (E₂) modulates SLC2A4/GLUT4 expression, but the involved mechanisms are unclear. Although E₂ exerts biological effects by binding to estrogen receptors 1/2 (ESR1/2), which are nuclear transcriptional factors; extranuclear effects have also been proposed. We hypothesize that E₂ regulates GLUT4 through an extranuclear ESR1 mechanism. Thus, we investigated the effects of E₂ upon (1) subcellular distribution of ESRs and the proto-oncogene tyrosine-protein kinases (SRC) involvement; (2) serine/threonine-protein kinase (AKT) activation; (3) Slc2a4/GLUT4 expression and (4) GLUT4 subcellular distribution and glucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were cultivated or not with E₂ for 24 h, and additionally treated or not with ESR1-selective agonist (PPT), ESR1-selective antagonist (MPP) or selective SRC inhibitor (PP2). Subcellular distribution of ESR1, ESR2 and GLUT4 was analyzed by immunocytochemistry; Slc2a4 mRNA and GLUT4 were quantified by qPCR and Western blotting, respectively; plasma membrane GLUT4 translocation and glucose uptake were analyzed under insulin stimulus for 20 min or not. E₂ induced (1) translocation of ESR1, but not of ESR2, from nucleus to plasma membrane and AKT phosphorylation, effects mimicked by PPT and blocked by MPP and PP2; (2) increased Slc2a4/GLUT4 expression and (3) increased insulin-stimulated GLUT4 translocation and glucose uptake. In conclusion, E₂ treatment promoted a SRC-mediated nucleus-plasma membrane shuttle of ESR1, and increased AKT phosphorylation, Slc2a4/GLUT4 expression and plasma membrane GLUT4 translocation; consequently, improving insulin-stimulated glucose uptake. These results unravel mechanisms through which estrogen improves insulin sensitivity.

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by disruption of glycemic homeostasis, involving impaired insulin-induced glucose uptake (Björnholm & Zierath 2005), in which reduced expression of the solute carrier family 2 of facilitated glucose transport member 4 (GLUT4) plays a fundamental role. This transporter,
encoded by the solute carrier family 2 member 4 gene (SLC2A4), is highly expressed in skeletal muscle and adipose tissue. GLUT4 translocates from intracellular microsomes to the cell surface in response to insulin, by means of a phosphatidylinositol 3-kinase (PI3K)- and serine/threonine-protein kinase (AKT)-mediated pathway (Tsuchiya et al. 2013), increasing glucose uptake in these tissues (Watson & Pessin 2001). Thus, although SLC2A4/GLUT4 expression is an essential element for tissue glucose disposal (Corrêa-Giannella & Machado 2013), it also depends on GLUT4 translocation, and disruption on this pathway may be related to impaired glucose uptake.

17β-estradiol (E2) is a steroid hormone, primarily known for its role in female physiology and reproduction. Nevertheless, E2 has played other physiological roles in both males and females, such as the control of glycemic homeostasis. Insulin resistance has been related to low estrogen levels in males (Smith et al. 1994, Faustini-Fustini et al. 1999); to both low and high estrogen levels in females (Solomon et al. 2001, Meyer et al. 2011) and to the pathogenesis of T2DM (Solomon et al. 2001, Meyer et al. 2011). Slc2a4/GLUT4 expression was observed to be reduced in muscle cells treated with 10−8M E2 for 24h (Barros et al. 2008), which could contribute to the E2-related insulin resistance; however, this effect in adipocytes is not clearly known.

Estrogens exert their main biological effects by binding to estrogen receptors 1 and 2 (ESR1 and ESR2), previously described as ERα and ERβ, respectively. ESRs are widely distributed throughout the body, exhibiting tissue- and cell-type-specific expression (Heldring et al. 2007, Nilson & Gustafsson 2011). In adipose tissue and skeletal muscle, both isoforms are expressed (Barros et al. 2006a, 2008, Campello et al. 2012a) in a distinct grade: ESR1 predominating in adipose tissue, whereas ESR2 in skeletal muscle (Barros et al. 2009).

The classical mechanism of E2 action occurs via nuclear ESRs that directly bind to specific estrogen-responsive elements (EREs) in the promoter region of target genes (Heldring et al. 2007). Moreover, nuclear ESRs can modulate the expression of genes lacking the EREs, through interaction with other transcriptional factors (Krishnan et al. 1994, Galien & Garcia 1997). Regarding that, NFkB has already been demonstrated as involved in the ESR1/ESR2-mediated modulation of the Slc2a4 gene expression in adipocytes (Campello et al. 2012a).

In addition to these well-established genomic actions, there is evidence that E2 also exerts extranuclear effects. These effects are mediated by (1) classical ESRs situated at or proximal to the plasma membrane (Levin 2014); (2) alternative spliced forms of ESR1 (ERα-46 and ERα-36) (Kang et al. 2010) and/or (3) G protein-coupled estrogen receptor (GPER1) (Prosnitz & Barton 2014). These extranuclear effects involve intracellular signals, such as activation of the PI3K/AKT pathway (Lucas et al. 2008, Garrido et al. 2013).

ESRs are known to be constantly shuttling between the cytoplasm and nucleus. Under steady-state conditions, ESRs have been predominantly detected in the nucleus, but the ESRs traffic in and out the plasma membrane has been described as well (Dauvois et al. 1993). A growing body of evidence demonstrates that the mechanisms responsible for ESR1 plasma membrane localization include posttranslational modifications of the receptor, such as palmitoylation and phosphorylation, followed by assembly of a protein complex with some membrane-associated proteins (Acconcia et al. 2005, Castoria et al. 2012, Adlanmerini et al. 2014). In this process, several proteins such as those from the SRC family (proto-oncogene tyrosine-protein kinases) are implicated. E2 can induce SRC-mediated phosphorylation of ESR1, thus inducing its nuclear exclusion and consequent translocation to plasma membrane (Lucas et al. 2008, Castoria et al. 2012).

On the other hand, the E2-induced ESR1 translocation to the plasma membrane and/or its surroundings has been described to promote ESR1/PI3K interaction, thus increasing AKT activity (Simoncini et al. 2000). Importantly, since PI3K/AKT participates in the insulin signaling pathway, plasma membrane ESR1 must play a significant role in insulin-sensitive tissues. In fact, modulations of insulin-induced glucose uptake in response to E2 have been described in adipocytes; however, in a controversial dose-dependent pattern (Collison et al. 2000, Muraki et al. 2006, Nagira et al. 2006). Additionally, there is evidence that ESR1-mediated E2 effect also alters the insulin receptor substrate IRS1 activity, upstream of the PI3K/AKT activation (Muraki et al. 2006, Nagira et al. 2006). However, contradictorily, the increased 10nM E2-induced IRS1/PI3K/AKT activity was observed concomitantly with ESR1 nuclear restraint (Nagira et al. 2006).

Altered skeletal muscle GLUT4 expression and glycemic homeostasis in ESR1−/− and ESR2−/− strongly suggests GLUT4 might be regulated by E2. Overall, it seems that E2, through an ESR1-mediated mechanism, might regulate insulin-induced glucose disposal in adipocytes; however, the accurate effect, as well as the GLUT4-related mechanisms, are yet unknown. Thus, this study attempted...
to investigate the effects of E₂ upon: (1) the subcellular distribution of ESRs and SRC protein involvement; (2) the AKT activation and (3) the GLUT4 subcellular distribution and consequent glucose uptake in 3T3-L1 adipocytes.

Materials and methods

3T3-L1 Cell culture

Mouse 3T3-L1 fibroblasts were obtained from American Type Culture Collection, Rio de Janeiro Cell Bank, Rio de Janeiro, RJ, Brazil (ATCC Number: CL-173TM). Cells were propagated and differentiated as previously described (Campello et al. 2012a, Furuya et al. 2013). Fully differentiated cells were starved in DMEM without phenol red (Sigma-Aldrich) and with FBS (10%) treated with dextran carbon (Sigma-Aldrich) for 24 h prior to treatment. Cells were subjected to differentiation at passages 8–10.

Cell treatments

Differentiated adipocytes were treated for 24 h in the absence (0E₂) or presence (10E₂) of 10 nM water-soluble 17β-estradiol (E₂, Sigma-Aldrich); added or not with (1) 10 nM of PPT, a selective ESR1 agonist (4,4',4''-(4-propyl-1H)-pyrazole-1,3,5-triyl)trisphenol; Sigma-Aldrich); (2) 1 µM of MPP, a selective ESR1 antagonist (1,3-bis(4-hydroxyphenyl)-4-methyl-5-(4-(2-piperidiny1ethoxy)phenol)-1H-pyrazole dihydrochloride; Tocris, Bristol, UK); (3) 10 nM of G15, a selective inhibitor of GPER1, ((3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline; Tocris) or (4) 10 nM PP2, a selective inhibitor of the SRC family of protein tyrosine kinases (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine; Calbiochem) (Konigame et al. 2011, Campello et al. 2012a). PPT and PP2 were solubilized in DMSO; then, when these compounds were used, DMSO was added to all experimental conditions at the same final concentration (0.1%). Additional studies were performed in cells starved from FBS in the last 3 h of treatments, to remove any insulin effect, and then stimulated or not with 17 nM insulin (Humulin R, Eli Lilly Brazil, São Paulo, Brazil) for 20 min (Campello et al. 2012a).

2-deoxi-D-[3H]glucose (2-DG) uptake

2-DG uptake assay was performed as previously described, and results were expressed as pmol/mg protein/min (Campello et al. 2012b).

Analysis of Slc2a4 mRNA and GLUT4 protein

Slc2a4 mRNA was analyzed by real-time PCR and GLUT4 protein by Western blotting, as previously described (Campello et al. 2012a). Total cellular GLUT4 was measured in cells homogenate, using actin cytoplasmic 1 protein (ACTB) as loading control. Additionally, subcellular fractionation was performed to obtain plasma membrane (PM)-enriched fraction, to quantify the GLUT4 translocation. The efficiency of this fractionation was previously confirmed: the PM fraction recovers ~85% of the total cellular 5’ nucleotidase activity, a plasma membrane marker (Campello et al. 2012b), and in vivo insulin-induced GLUT4 increase in PM and decrease in microsomal fraction (M) were clearly observed by Western blotting. The Ponceau-stained nitrocellulose membranes were used as loading control for PM and M GLUT4 measurement (Romero-Calvo et al. 2010).

Analysis of Esr1 and Esr2 mRNA

Differentiated 3T3-L1 cells were lysed in TRIzol reagent (Thermo Scientific, #15596). Total RNA was extracted with phase separation reagent (BCP) (MRC, #151) and RTNeasy kit (Qiagen, #74104), and reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, #4387406), according to manufacturer’s instructions. TaqMan Universal Mastermix II (Applied Biosystems, #4440038) and assays Mm00433149_m1 and Mm00599821_m1 were used for amplification of Esr1 and Esr2; respectively, in a QuantStudio 12K Flex Real-Time PCR System machine. Relative expression values were calculated from the threshold cycle (Ct) following the 2−∆∆CT method and using Gapdh (Mm99999915_g1) as an internal reference gene.

ESR1/2 and GLUT4 cellular localization

Immunofluorescence analysis of ESR1, ESR2 and GLUT4 was performed as previously described (Barros et al. 2006b, Lucas et al. 2008). Briefly, 3T3-L1 cells were grown and differentiated on glass coverslips coated with gelatin (0.1%). After blocking, cells were incubated with one of the following primary antibodies: rabbit polyclonal antibody anti-ESR1 (MC-20, Santa Cruz Biotechnology) diluted at 1:50, goat polyclonal antibody anti-ESR2 (L-20, Santa Cruz) diluted at 1:50 and rabbit polyclonal anti-GLUT4 antibody (07-1404, EMD Millipore) diluted at 1:100, in PBS containing 0.01% saponin and 1% BSA.
for 1 h at room temperature. The appropriate Alexa Fluor 594-labeled secondary antibody (Molecular Probes) was used at a final concentration of 1:300. Afterwards, the slides were incubated with nuclear marker (DAPI) (Sigma-Aldrich) and wheat germ agglutinin (WGA) (Sigma-Aldrich). Negative control slides were analyzed in the absence of primary antibody. Images were obtained as previously described (Lucas et al. 2008).

**Total AKT and phospho-AKT protein analysis**

Total AKT and phospho-AKT were measured in a cellular lysate as previously described (De Castro Barbosa et al. 2013). Membranes were firstly probed with antibody anti p-AKT1/2/3 (p-AKT1/2/3, sc-7985-R, Santa Cruz Biotechnology); secondly, reprobed with antibody anti-total AKT1/2/3 (AKT1/2/3-H-136, sc-8312, Santa Cruz Biotechnology), and finally, reprobed with antibody anti-actin cytoplasmic 1 protein (ACTB) (monoclonal anti-beta-actin, A2228, Sigma-Aldrich). To analyze insulin-stimulated cells, 15 µg of protein was loaded for electrophoresis, whereas to analyze cells not exposed to insulin, 50 µg of protein was loaded. Results of total AKT and p-AKT were firstly normalized by the internal control ACTB; thus, p-AKT was related to total AKT.

**Data analysis**

Results were expressed as mean±standard error of mean (s.e.m.) and compared by two-way ANOVA (Bonferroni post-test) or by one-way ANOVA (Newman–Keuls post-test) as indicated. When only two groups were compared, parametric (unpaired Student t) or nonparametric (Mann–Whitney) tests were used by comparing the variances, as indicated in legends of figures.

**Results**

**E₂ enhances *Slc2a4/GLUT4* expression**

To investigate the effects of E₂ upon glucose disposal in adipocytes, we firstly investigated the glucose transporter expression (Fig. 1A and B). Treatment of 3T3-L1 adipocytes with 10nM E₂ for 24 h increased both *Slc2a4* mRNA and the total cellular GLUT4 protein (*P*<0.001 vs 0E₂).

**E₂ stimulates GLUT4 translocation to plasma membrane and increases insulin-induced glucose uptake**

GLUT4 subcellular localization was initially analyzed by immunocytochemistry. As shown in Fig. 1C, insulin induced an evident GLUT4 translocation to the plasma membrane in the absence of E₂ (0E₂ (+)). Surprisingly, the plasma membrane GLUT4 content also increased in response to E₂, even in the absence of insulin (10E₂ (−)), and that was further enhanced in the presence of insulin (10E₂ (+)).

To quantify the GLUT4 translocation, Western blotting analysis of GLUT4 in plasma membrane fraction was performed (Fig. 2A and B). Plasma membrane GLUT4 content was clearly increased by insulin and E₂. Two-way ANOVA revealed that both E₂ (*P*=0.0006) and insulin (*P*<0.0001) have independent (no interaction *P*=0.6022) positive effects upon GLUT4 translocation to PM (Fig. 2B). Furthermore, the Bonferroni post-test confirmed that 10E₂ treatment increased (*P*<0.05) the GLUT4 translocation to plasma membrane independently of the insulin stimulus.

Thus, the 2-DG uptake was investigated (Fig. 2C), and the results were in accordance with the GLUT4 subcellular distribution. Two-way ANOVA revealed that cellular glucose disposal increased in response to both E₂ (*P*=0.0214) and insulin (*P*<0.0001) and that there is no interaction between these variables (*P*=0.1062). However, the Bonferroni post-test revealed a significant 10nM E₂ effect on 2-DG uptake only under the insulin-stimulated condition (*P*<0.05).

**E₂ induces ESR1 translocation to plasma membrane**

Figure 3 shows the subcellular localization of ESR1 (Fig. 3A) and ESR2 (Fig. 3B). Under basal condition, that is, in the absence of E₂ (0E₂), we detected specific staining for ESR1 and ESR2 mainly in the nuclei of 3T3-L1 adipocytes. After 24-h treatment with 10nM E₂ (10E₂), ESR1 was highly detected in plasma membranes, as confirmed by the co-localization of ESR1 and WGA (plasma membrane marker). This E₂ effect was not observed for ESR2, which clearly remained in the nuclei. These results show that the ESR1, but not ESR2, translocates from nucleus to plasma membrane upon E₂ treatment of adipocytes.

We also examined the relative expression of *Esr1* and *Esr2* mRNAs in differentiated 3T3-L1 adipocytes. As we can observe (Fig. 3C), *Esr1* mRNA content was 10-fold higher than *Esr2* mRNA content, suggesting that the ESR1 protein is more abundant in this cell.

**Translocation of ESR1 is also induced by PPT and blocked by MPP and PP2**

Figure 3D shows that PPT (ESR1-selective agonist) could induce ESR1 translocation to plasma membrane, in the
absence or presence of E$_2$, mimicking the E$_2$ effect. Besides, Fig. 3E shows that MPP (ESR1-selective antagonist) abrogated the E$_2$-induced plasma membrane translocation of ESR1. These results reveal that ESR1 redistribution was dependent on its own activity.

We also tested the involvement of SRC protein kinases in ESR1 translocation, from nucleus to plasma membrane (Fig. 3F), by concomitant treatment of the adipocytes with E$_2$ and PP2, a selective inhibitor of SRC kinases. The addition of PP2 completely blocked the E$_2$-induced ESR1 translocation, evincing the participation of SRC proteins in E$_2$-induced ESR1 redistribution.

**Insulin causes no acute change in ESR1 translocation**

Twenty-minute insulin stimulation, at the end of the 24-h E$_2$ treatment (Fig. 4A), did not alter the subcellular distribution of ESR1 caused by E$_2$, nor the distribution caused by PPT (Fig. 4B), MPP (Fig. 4C) and PP2 (Fig. 4D), compared with the effects described in the absence of insulin (Fig. 3A, B, D, E and F; respectively).

**Plasma membrane ESR1 translocation is accompanied by increased AKT activity**

Some effects of plasma membrane-related ESR1 have been described as mediated by AKT, a key step in the insulin signaling pathway. Considering that, we evaluated AKT phosphorylation under conditions in which ESR1 is translocated from nucleus to plasma membrane. Figure 5A shows that 24-h 10 nM E$_2$ induced a ~20-fold increase in AKT phosphorylation (P<0.05), pointing out the absence of insulin. Furthermore, in zero E$_2$ and zero insulin, the
sensitized the adipocytes to the insulin-stimulated AKT phosphorylation (E$_2$: $P<0.0001$, two-way ANOVA); PPT: $P<0.001$ vs 0E$_2$); besides, MPP abrogated the E$_2$-induced effect ($P<0.001$ vs 10E$_2$). Finally, Fig. 6B shows that E$_2$-induced enhancement of insulin-stimulated AKT phosphorylation was also abrogated by PP2 ($P<0.001$ vs 10E$_2$).

G protein-coupled estrogen receptor (GPER1) does not participate in the E$_2$-induced activation of AKT

To check the probable participation of GPER1 in the E$_2$ sensitizer effect of insulin-stimulated activation of AKT, we treated the cells with 10E$_2$ plus G15, an inhibitor of GPER1. Figure 7 shows that G15 did not alter the E$_2$ activation of AKT, indicating that the E$_2$ effect occurs independently of GPER1.

Discussion

Estrogen is reported to participate in the glycemic homeostasis, and E$_2$-induced modulation of cellular glucose disposal has been proposed since the beginning of years 2000 (Barros et al. 2006a). Recently, in MCF-7 cells, a rapid (25 min) enhancing effect of E$_2$ upon glucose uptake was reported and suggested to be related to the plasma membrane ESR1 localization (Garrido et al. 2013). Here, we unravel a plasma membrane ESR1-related E$_2$ stimulation of glucose uptake, in a classic insulin-sensitive cell.

We observed that Esr1 mRNA expression is higher than Esr2 mRNA expression in differentiated 3T3-L1 adipocyte; similar to that described in differentiated human adipocytes (Dieudonné et al. 2004). The Esr1>Esr2 mRNA expression, analyzed by quantitative real-time PCR, offers accurate data, which allow us to suggest that ESR1 protein is also more abundant than ESR2 protein. However, that cannot be confirmed, since the effectiveness of different antibodies (anti-ESR1 vs anti-ESR2) is unmatched.

An important issue is the hypothetical effect of E$_2$ upon ESR1/ESR2 expression. Human adipocytes, subjected to 24-h culture with 100nM E$_2$, alter ESR1/ESR2 mRNA expression according to the adipose depot (subcutaneous or intra-abdominal) and to the gender (male or female); however, the respective proteins were not evaluated (Dieudonné et al. 2004). In previous study, we reported that ESR1 and ESR2 protein contents do not change in response to 24-h 10nM E$_2$ (Campello et al. 2012a); thus, it becomes clear that E$_2$ acts in 3T3-L1 adipocytes mainly

Figure 2

E$_2$ stimulates GLUT4 translocation and increases insulin-induced glucose uptake. Adipocytes were cultivated in the absence (0E$_2$) and presence of 10nM E$_2$ (10E$_2$) for 24 h, and additionally insulin stimulated (+) during the last 20 min or not (-). (A) Representative GLUT4 protein immunoblots in PM and respective loading control in the Ponceau-stained membrane. (B) PM GLUT4 translocation and (C) 2-deoxy-D-[3H]glucose (2-DG) uptake. Results are means ± s.e.m. of 5 samples and were analyzed by two-way ANOVA: in (B), $P=0.0006$ for E$_2$, $P=0.0040$ for insulin and $P=0.6022$ for interaction and in (C), $P=0.0214$ for E$_2$, $P<0.0001$ for insulin, $P=0.1062$ for interaction. *$P<0.05$ and **$P<0.01$ vs respective condition in 0E$_2$ (Bonferroni post-test).

p-AKT content is very low, although clearly detected by optic densitometry; that is the reason why we considered 10E$_2$ as 100.

Additionally (Fig. 5B), we detected, always in the absence of insulin, that the ESR1-agonist PPT mimicked 10E$_2$ effect and that the SRC kinases inhibitor PP2 completely blocked the 10E$_2$ effect.

Once the effects of E$_2$, PPT and PP2 on AKT phosphorylation in the absence of insulin were demonstrated, additional experiments were performed in the presence of insulin. E$_2$ (10nM) and PPT (Fig. 6A)
ESR1-mediated E₂ effect on GLUT4 protein via ESR1, involving ESR1 subcellular redistribution. Nevertheless, the present results, obtained in a cell line originated from a mouse embryo, must be carefully transposed to humans.

The main biological effects of E₂ are mediated by ESRs; however, in addition to their classical nuclear action, recent studies have shown that ESRs also act outside the nucleus (Gutiérrez et al. 2012). ESR1 and ESR2 translocation to plasma membrane was described as a rapid 10- to 15-min response (Lucas et al. 2008, Bondar et al. 2009, Gutiérrez et al. 2012). In 3T3-L1 adipocytes, plasma membrane translocation of ESR1 was observed after 16-h E₂ treatment (Nagira et al. 2006), but with a hormone concentration one thousand higher than that used in the present study. For the first time, we show a remarkable E₂-induced selective ESR1 shuttle from nucleus to plasma membrane, after a 24-h treatment with 10nM E₂; an effect that was mimicked by PPT (ESR1 agonist) and abrogated by MPP (ESR1 antagonist). These data reveal that the activation of
ESR1 is necessary for its own subcellular redistribution in 3T3-L1 adipocytes.

The SRC tyrosine kinases present several functional domains. When the Tyr S30 in the C-terminus is dephosphorylated, the kinase activity is triggered, and the protein interacts with target proteins (Chojnacka & Mruk 2015). ESR1-Tyr537 phosphorylation induces its molecular interaction with SRC protein (Nieto et al. 2015), leading to SRC activation (Barletta et al. 2004); on the other hand, ESR1-Tyr537 phosphorylation has been...
ESR1-mediated E\(_2\) effect on GLUT4 protein

**ESR1-mediated E\(_2\) effect on GLUT4 protein**

Proposed to occur in response to SRC activation, leading to nuclear shuttle of the receptor (Castoria et al. 2012). In fact, in Sertoli cells, 10-min E\(_2\) induces a SRC-mediated translocation of both ESR1 and ESR2 to plasma membrane (Lucas et al. 2008). Our results in adipocytes revealed that the SRC inhibitor (PP2) blocked the 24-h E\(_2\)-induced ESR1 translocation from nucleus to membrane, confirming the participation of SRC proteins in the ESR1 mobilization.

E\(_2\)-induced activation of PI3K/AKT was clearly reported to occur via ESR1 in MCF-7 cells (Lee et al. 2005). In 3T3-L1 adipocytes, 100nM E\(_2\) (high concentration) rapidly activates IRS1 and AKT, but the participation of ESR1 or ESR2 was not investigated (Kim et al. 2012). Regarding that, plasma membrane-related ESR1 was reported to bind and activate PI3K in endothelial cells (Simoncini et al. 2000), thus explaining AKT activation, which could enhance the insulin effect on this pathway. In fact, 10nM E\(_2\) enhanced the insulin-stimulated activation of AKT and glucose uptake in adipocytes; however, that...
was inexplicably related to low plasma membrane and high nuclear contents of ESR1 (Nagira et al. 2006). Those studies together do not provide a clear comprehension of E2-induced plasma membrane translocation of ESR1, AKT activation and cellular glucose disposal improvement.

To unravel those E2-induced effects, we firstly investigated the AKT activation in the absence of insulin, seeking to relate the results to the plasma membrane ESR1 localization. We demonstrated that 10 nM E2 induced a strong increase in AKT phosphorylation, an effect that was mimicked by PPT and abrogated by PP2. These data together relate the AKT activation to the plasma membrane ERS1 localization.

Besides, E2 was also analyzed as a sensitizer of insulin-induced stimulation of AKT, since, in vivo, insulin plays a fundamental role in AKT activity. E2 and PPT increased the insulin-stimulated AKT phosphorylation, and the E2 effect was blocked by MPP or PP2. These data disclose E2 as a sensitizer of the insulin-stimulated activation of AKT and relate the E2 effect to the plasma membrane ESR1 translocation.

Finally, considering that E2 also triggers GPER1 activation, and that this receptor might also activate AKT, we tested the effect of GPER1 inhibitor G15. The results showed that G15 did not alter the E2 sensitizer effect on insulin-stimulated AKT phosphorylation. In fact, participation of GPER1 in the E2-induced activation of AKT is variable, according to the cell type (Vivacqua et al. 2006, Carnesecchi et al. 2015). In summary, the absence of G15 effect reinforces the relationship between plasma membrane ESR1 and AKT activation.

AKT plays a fundamental role in glucose uptake in insulin-sensitive tissues, participating in the insulin-stimulated translocation of GLUT4 storage vesicles (GSV) from the intracellular to the plasma membrane (Kohn et al. 1996). On the other hand, it is known that increased plasma membrane GLUT4 content naturally accompanies increased Slc2a4/GLUT4 expression, as demonstrated in transgenic mice that overexpress GLUT4 (Gibbs et al. 1995). In the present study, ESR1-mediated E2 effect increased PM GLUT4 content by AKT-mediated GSV translocation. Besides, E2 increased the Slc2a4/GLUT4 expression, which additionally contributes to increase PM GLUT4, and also seems to be related to ESR1, since ESR1 activity increases, whereas ESR2 activity decreases Slc2a4/GLUT4 expression (Campello et al. 2012a).

The E2-stimulated Slc2a4 expression has been proposed as an ESR1-mediated genomic effect on the promoter region of Slc2a4 gene, in which the repression of NFKB could participate (Barros et al. 2006a). Now, the present data depict that E2 induces a strong nuclear shuttle of ESR1, indicating that the enhancement of Slc2a4 expression could be triggered outside the nucleus, despite involving the NFKB pathway. To support this hypothesis, we point out that: (1) AKT activation inhibits NFKB activity (Ruan et al. 2002), (2) NFKB is a powerful repressor of Slc2a4 transcription (Furuya et al. 2013) and (3) insulin-induced activation of AKT reduces NFKB-mediated repression of Slc2a4 transcription (Moraes et al. 2014). Furthermore, in 3T3-L1 adipocytes, we had already reported that 24-h PPT treatment reduces NFKB binding to Slc2a4 gene, enhancing Slc2a4/GLUT4 expression (Campello et al. 2012a), which now became clear to be triggered by the plasma membrane-related ESR1. Additionally, the present study confirmed an ESR1-mediated E2 effect upon glucose uptake in adipocytes; however, for the first time, this effect is clearly related to the plasma membrane localization of ESR1, AKT activation and GLUT4 translocation under the same experimental protocol.

An unexpected result was observed in response to E2 in the absence of insulin: despite the increased PM GLUT4 translocation, the glucose uptake did not increase. It has been proposed that in addition to AKT-dependent phosphorylation of AS160, insulin-induced activation of some plasma membrane- and actin remodeling-related proteins is also required for GSV docking and fusion to the cell surface (Koumanov et al. 2005, Bai et al. 2007). Thus, we can propose that E2 induces the translocation of GSV to the plasma membrane, but the GSV docking and/or fusion are not achieved in the absence of insulin, preventing the increase in basal glucose uptake.

A growing body of evidence indicates a relationship between E2 and glycemic homeostasis in vivo. In this regard, E2 treatment has been shown to restore the glucose uptake in oophorectomized rats (Kumagai et al. 1993) and to maintain the insulin sensitivity in postmenopausal women (Moreno et al. 2010). In the present study, we provide evidence that 24-h E2 treatment of 3T3-L1 adipocytes induces a SRC-mediated nucleus-plasma membrane shuttle of ESR1, which is accompanied by increased phosphorylation of AKT, and increased Slc2a4/GLUT4 expression and plasma membrane GLUT4 translocation; consequently, improving insulin-stimulated glucose uptake. These results unravel mechanisms through which estrogen contributes to improve insulin sensitivity, highlighting that this effect is mediated by ESR1.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
Funding
This research was supported by FAPESP (São Paulo State Foundation for Research) grants 2012/24210-1, 2012/04831-1 and 2016/15603-1.

Acknowledgment
The authors thank Dr Adauri Brezolin for revision of the English language of this manuscript.

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ESR1-mediated E\(_2\) effect on GLUT4 protein


Received in final form 7 June 2017
Accepted 20 July 2017
Accepted Preprint published online 20 July 2017