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 (E_2) modulates *SLC2A4*/GLUT4 expression, but the involved mechanisms are unclear. Although E_2 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 E_2 regulates GLUT4 through an extranuclear ESR1 mechanism. Thus, we investigated the effects of E_2 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 E_2 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. E_2 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, E_2 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.

Key Words

- ▶ extranuclear ESR1
- ▶ *Slc2a4*
- ▶ SRC
- ▶ PPT
- ▶ MPP
- ▶ PP2

*Journal of Molecular
Endocrinology*
(2017) **59**, 257–268

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 microsome 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.

17 β -estradiol (E_2) is a steroid hormone, primarily known for its role in female physiology and reproduction. Nevertheless, E_2 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 E_2 for 24 h (Barros *et al.* 2008), which could contribute to the E_2 -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 ER α and ER β , respectively. ESRs are widely distributed throughout the body, exhibiting tissue- and cell-type-specific expression (Heldring *et al.* 2007, Nilsson & 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 E_2 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, NF κ B 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 E_2 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 (ER α -46 and ER α -36) (Kang *et al.* 2010) and/or (3) G protein-coupled estrogen receptor (GPER1) (Prossnitz & 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. E_2 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 E_2 -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 E_2 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 E_2 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 10 nM E_2 -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 E_2 . Overall, it seems that E_2 , 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 E_2 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 24h prior to treatment. Cells were subjected to differentiation at passages 8–10.

Cell treatments

Differentiated adipocytes were treated for 24h in the absence ($0E_2$) or presence ($10E_2$) of 10nM water-soluble 17 β -estradiol (E_2 ; 389; Sigma-Aldrich); added or not with (1) 10nM of PPT, a selective ESR1 agonist (4,4',4''-(4-propyl-1H)-pyrazole-1,3,5-triyl)trisphenol; Sigma-Aldrich); (2) 1 μ M of MPP, a selective ESR1 antagonist (1,3-bis (4-hydroxyphenyl)-4-methyl-5-(4-(2-piperidinylethoxy) phenol)-1H-pyrazole dihydrochloride; Tocris, Bristol, UK); (3) 10nM 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) 10nM 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 3h 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 20min (Campello *et al.* 2012a).

2-deoxy-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 RNeasy 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 $2^{-\Delta\Delta CT}$ 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, re-probed with antibody anti-total AKT1/2/3 (AKT1/2/3-H-136, sc-8312, Santa Cruz Biotechnology), and finally, re-probed with antibody anti-actin cytoplasmic 1 protein (ACTB) (monoclonal anti-beta-actin, A2228, Sigma-Aldrich). To analyze insulin-stimulated cells, 15 μ g of protein was loaded for electrophoresis, whereas to analyze cells not exposed to insulin, 50 μ g 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 $\pm$ 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

E_2 enhances *Slc2a4*/GLUT4 expression

To investigate the effects of E_2 upon glucose disposal in adipocytes, we firstly investigated the glucose transporter expression (Fig. 1A and B). Treatment of 3T3-L1 adipocytes with 10 nM E_2 for 24 h increased both *Slc2a4* mRNA and the total cellular GLUT4 protein ($P < 0.001$ vs $0E_2$).

E_2 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 E_2 ($0E_2$ (+)). Surprisingly, the plasma membrane GLUT4 content also increased in response to E_2 , even in the absence of insulin ($10E_2$ (-)), and that was further enhanced in the presence of insulin ($10E_2$ (+)).

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 E_2 . Two-way ANOVA revealed that both E_2 ($P = 0.0006$) 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 $10E_2$ 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 E_2 ($P = 0.0214$) 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 10 nM E_2 effect on 2-DG uptake only under the insulin-stimulated condition ($P < 0.05$).

E_2 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 E_2 ($0E_2$), we detected specific staining for ESR1 and ESR2 mainly in the nuclei of 3T3-L1 adipocytes. After 24-h treatment with 10 nM E_2 ($10E_2$), ESR1 was highly detected in plasma membranes, as confirmed by the co-localization of ESR1 and WGA (plasma membrane marker). This E_2 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 E_2 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

Figure 1

E_2 enhances *Slc2a4*/GLUT4 expression and GLUT4 translocation. Adipocytes were cultivated in the absence ($0E_2$) and presence of 10 nM E_2 ($10E_2$) for 24 h to analyze *Slc2a4* mRNA (A) and total cellular GLUT4 (B) contents. Representative immunoblots of GLUT4 and ACTB (loading control) are shown (B). Data are means $\pm$ s.e.m. of 5–8 samples, analyzed by Mann–Whitney test (A) or unpaired Student *t* test (B). ** $P < 0.01$ and *** $P < 0.001$ vs $0E_2$. For GLUT4 translocation analysis (C), cultivated cells were insulin stimulated (+) for 20 min or not (–). Immunofluorescence shows GLUT4 (red), nuclear marker DAPI (blue) and plasma membrane marker WGA (green). Merged images show the plasma membrane GLUT4 localization (yellow). Images are representative of at least 3 independent experiments. Scale bar is indicated.

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

Figure 2

E_2 stimulates GLUT4 translocation and increases insulin-induced glucose uptake. Adipocytes were cultivated in the absence ($0E_2$) and presence of 10 nM 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 $\pm$ 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 (10 nM) and PPT (Fig. 6A)

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 100 nM 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 10 nM E_2 (Campello *et al.* 2012a); thus, it becomes clear that E_2 acts in 3T3-L1 adipocytes mainly

Figure 3

Subcellular distribution and gene expression of ESR1 and ESR2. Panels (A) and (B) show the subcellular localization of ESR1 and ESR2; respectively, in adipocytes cultivated in absence (0E₂) or presence of 10 nM E₂ (10E₂) for 24 h. Relative expression (C) of *Esr1* and *Esr2* mRNAs was analyzed using *Gapdh* gene as internal control; *** $P < 0.0001$. Subcellular localization of ESR1 in adipocytes is also shown in adipocytes treated with ESR1-agonist PPT (D), ESR1-antagonist MPP (E) or SRC inhibitor PP2 (F), in the absence (0E₂) or presence of 10 nM E₂ (10E₂), for 24 h. Immunofluorescence shows ESR1 and ESR2 (red), nuclear marker DAPI (blue) and plasma membrane marker WGA (green). Merged images show the nuclear localization of ESR1 and ESR2 (magenta), and the plasma membrane localization of ESR1 (yellow). Images are representative of at least 3 independent experiments. Scale bars (20 μ m) are indicated.

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 10 nM E₂; an effect that was mimicked by PPT (ESR1 agonist) and abrogated by MPP (ESR1 antagonist). These data reveal that the activation of

Figure 4

Insulin stimulation did not alter redistribution of ESR1. Subcellular localization of ESR1 in 3T3L1 adipocytes 24h treated with zero ($0E_2$) or 10 ($10E_2$) nM 17β -estradiol (A), $0E_2$ and $10E_2$ +SR1 agonist PPT (B), $0E_2$ and $10E_2$ +SR1 antagonist MPP (C); $0E_2$ and $10E_2$ +SRC antagonist PP2 (D). Under all conditions, the cells were stimulated with insulin (20 min) at the end of the 24 h treatment. Images show immunofluorescence of ESR1 (red), nuclear marker DAPI (blue) and plasma membrane marker WGA (green). Merged images show ESR1 localization in nucleus (magenta) or in plasma membrane (yellow). Images are representative of at least 3 independent experiments. Scale bars (20 μ m) are indicated.

Figure 5

Plasma membrane ESR1 translocation is accompanied by increased AKT activity. Adipocytes were cultivated in absence of 10 nM E_2 ($0E_2$), or in the presence of E_2 ($10E_2$), ESR1-selective agonist (PPT), or $10E_2$ plus SRC antagonist ($10E_2$ +PP2), and not stimulated with insulin. In (A), $10E_2$ effect was investigated separately, whereas in (B), it was compared to PPT and PP2 effects. In each panel, representative images of p-AKT, total AKT and ACTB are shown. Results of p-AKT were normalized by ACTB as loading control, and thus by the results of total AKT. Results are means $\pm$ s.e.m. of 4 (A) and 5 (B) samples. Data were analyzed by Mann-Whitney test in (A), $*P < 0.05$; and by one-way ANOVA, followed by Newman-Keuls post-test, in (B): $***P < 0.001$ vs $0E_2$, $###P < 0.01$ vs $10E_2$ and $\&\&\&P < 0.01$ vs PPT.

ESR1 is necessary for its own subcellular redistribution in 3T3-L1 adipocytes.

The SRC tyrosine kinases present several functional domains. When the Tyr 530 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

Figure 6

Plasma membrane ESR1 translocation sensitizes insulin-induced AKT activation. Adipocytes were cultivated in absence (0E₂) or in presence of 10nM E₂ (10E₂), and additionally treated or not with ESR1 agonist (PPT) and ESR1 antagonist (MPP), panel (A), or with SRC antagonist (PP2), panel (B). Under all conditions, the cells were stimulated with insulin (20 min) at the end of the 24-h treatment. In each panel, representative images of p-AKT, total AKT and ACTB are shown. Results of p-AKT were normalized by ACTB as loading control, and thus by the results of total AKT. Results are means $\pm$ S.E.M. of 5–8 (A) or 5 samples (B). Data were analyzed by two-way ANOVA: $P < 0.0001$ for E₂, $P < 0.01$ for treatments (PPT, MPP and PP2) and $P < 0.01$ for interaction, *** $P < 0.001$ vs respective untreated condition (Bonferroni post-test).

Figure 7

E₂ sensitization of insulin-induced activation of AKT is not mediated by GPER1. Adipocytes were cultivated in the absence (0E₂) or in presence of 10nM E₂ (10E₂) and treated or not with GPER1 antagonist (G15). Additionally, the cells were stimulated with insulin (20 min) at the end of the 24-h treatment. Representative images of p-AKT, total AKT and ACTB are shown. Results of p-AKT were normalized by ACTB as loading control, and thus, by the results of total AKT. Results are means $\pm$ S.E.M. of 6 samples and were analyzed by two-way ANOVA: $P < 0.0001$ for E₂, *** $P < 0.001$ vs respective 0E₂ (Bonferroni post-test).

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 E₂ 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 E₂-induced ESR1 translocation from nucleus to membrane, confirming the participation of SRC proteins in the ESR1 mobilization.

E₂-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 E₂ (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 E₂ 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 E_2 -induced plasma membrane translocation of ESR1, AKT activation and cellular glucose disposal improvement.

To unravel those E_2 -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 E_2 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 ESR1 localization.

Besides, E_2 was also analyzed as a sensitizer of insulin-induced stimulation of AKT, since, *in vivo*, insulin plays a fundamental role in AKT activity. E_2 and PPT increased the insulin-stimulated AKT phosphorylation, and the E_2 effect was blocked by MPP or PP2. These data disclose E_2 as a sensitizer of the insulin-stimulated activation of AKT and relate the E_2 effect to the plasma membrane ESR1 translocation.

Finally, considering that E_2 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 E_2 sensitizer effect on insulin-stimulated AKT phosphorylation. In fact, participation of GPER1 in the E_2 -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 E_2 effect increased PM GLUT4 content by AKT-mediated GSV translocation. Besides, E_2 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 E_2 -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 E_2 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 E_2 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 E_2 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 E_2 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 E_2 and glycemic homeostasis *in vivo*. In this regard, E_2 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 E_2 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 Aduari Brezolin for revision of the English language of this manuscript.

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Received in final form 7 June 2017

Accepted 20 July 2017

Accepted Preprint published online 20 July 2017