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ESR1-Mediated Regulation of *Slc2a4* Gene Expression by Estrogen: Demonstration of a Potential Estrogen Responsive Element (ERE) in the *Slc2a4* Gene Promoter

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Abstract: This study investigated the transcriptional activity (gene reporter assay) of some putative estrogen responsive elements (EREs) in the *Slc2a4* gene promoter region, which could contribute to understanding the estrogen receptor 1 (ESR1) mediated enhancer role of estradiol (E2). In 3T3L1 adipocytes, E2 and ESR1-selective agonist (PPT) increased (by 35%, $p<0.01$) the expression of *Slc2a4* mRNA; ESR1-selective antagonist (MPP) abrogated the E2 effect. In transiently transfected HEK293-FT cells, the basal *Slc2a4*-Luciferase activity of 5'-500, 5'-250 and 5'-149 bp segments of the promoter revealed that the loss of the -500/-250 bp segment reduces the unstimulated *Slc2a4*-Luc activity (60%, $p<0.001$), a response attributed to the loss of seven known binding-sites of enhancer transcription factors. Besides, E2/PPT increased (~150%, $p<0.001$) the relative *Slc2a4*-Luc activity of all constructs, revealing that the ESR1-mediated E2-induced transcriptional activity is preserved in all constructs, and that the putative ERE half-sites, encompassed in -250/-149 bp segment, do not display transcriptional activity. Finally, 5'-500 bp and 5'-149 bp constructs, containing four mutated nucleotides in the putative complete *Slc2a4* ERE, lost their responsiveness to E2. In conclusion, this study reveals an imperfect complete *Slc2a4* ERE site (-59/-46 bp), which is involved in the ESR1-mediated E2-induced enhancing of the *Slc2a4* gene expression.

Keywords: diabetes mellitus, insulin resistance, GLUT4, 17β-estradiol, PPT, estrogen receptor.

INTRODUCTION

More than half a century ago the estrogen-induced improvement of glycemic control started to be reported in hypoestrogenic women [1-4]. Since then, both hypoestrogenism and hyperestrogenism have been associated with insulin resistance (IR) and impaired glycemic homeostasis in women [1-3,5-7] and men [8-11].

The glucose transporter protein GLUT4 (solute carrier family 2, facilitated glucose transporter member 4), codified by the *Slc2a4* (solute carrier family 2 member 4) gene, is responsible for insulin-stimulated glucose uptake in muscle and adipose tissues [12], thus participating in the glycemic homeostasis [13]. Reduced expression of the *Slc2a4*/GLUT4 is a feature in IR, even under preserved insulin-stimulated translocation [14], depicting the importance of the reduced *Slc2a4*/GLUT4 expression in the chronic IR condition such as type

2 diabetes (T2D). Estrogen mechanism of action is mainly mediated by two estrogen receptors, ESR1 (estrogen receptor 1; formerly ER alpha) and ESR2 (estrogen receptor 2; formerly ER beta), codified by genes *ESR1* and *ESR2*, and belong to the nuclear receptor family, thus playing a role of transcription factor [15,16]. Although ESR1 and ESR2 have similar affinity to 17β-estradiol (E2), and bind into the same estrogen responsive element (ERE) in the DNA of target genes, they display variable capacity to recruit a range of co-regulatory protein complexes, and that may greatly change their final biological effect [16].

Classical mechanism of ESRs binding has been described to occur as homo- or heterodimers into a ERE binding-site [15,16], such as the perfect palindromic ERE consensus sequence AGGTCANNNTGACCT, and also in imperfect ERE sequences; besides ESRs can bind, usually as a monomer, in perfect/imperfect ERE half-sites [17-20]. Moreover, in monomeric binding, secondary protein interaction is facilitated, favoring an estrogen-induced cooperative mechanism involving one ESR monomer bound into one ERE half-site and other transcriptional factor bound in its respective responsive element nearby [15,18,21].

Since the remarkable studies investigating the GLUT4 expression in skeletal muscle of ESR1$^{-/-}$ and ESR2$^{-/-}$ mice [22,23], ESRs have made a great contribution to the understanding of the glycemic homeostasis regulation by estrogen. Those studies have revealed that ESR1 enhances, whereas ESR2 represses, the *Slc2a4*/GLUT4 expression; and changes in the amount of each receptor reflects in glycemic homeostasis: ESR1$^{-/-}$ mice show IR and develop T2D, whereas ESR2$^{-/-}$ mice exhibit increased insulin sensitivity with fasting hypoglycemia [23-25]. Furthermore, it was clearly reported that the ESR2 is much more abundant in skeletal muscle, whereas ESR1 is predominant in adipose tissue, illustrating the complex participation of ESRs in the metabolic network [25].

Once the GLUT4 plays an important role in glycemic control, the *SLC2A4* gene has become a promising target for pharmacogenomics of IR [26], and transcriptional regulation of the gene expression has been investigated [27]. Several transcriptional factors have already been reported as modulators of the expression of *SLC2A4*/*Slc2a4* (human/murine genes, respectively), most of them acting as enhancers and a few as repressors [28,29]; besides, their DNA-binding sites in the promoter of the *SLC2A4*/*Slc2a4* gene were also identified [30-38]. Nevertheless, despite functional demonstration of ESRs-mediated E2-induced regulation of the *Slc2a4* gene expression, the genomic mechanisms involved are unclear. *In-silico* analysis of the 5'-400 bp segment of the *Slc2a4* promoter identified five putative half-sites of ERE (60%-67% homology with the consensus ERE half-sites) and one putative complete ERE (73% homology with the consensus complete ERE) [27]. However, the participation of these EREs domains in the *Slc2a4* expression has never been investigated. Thus, the aim of the present study is to demonstrate the transcriptional activity of a putative imperfect complete ERE sequence in the *Slc2a4* promoter, in which ESR1 must play an enhancer role, acting as a cis-regulatory element.

MATERIALS AND METHODS

Cell Cultures and Treatments

Mouse 3T3-L1 fibroblasts, obtained from the American Type Culture Collection (ATCC®: #CL-173TM, Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil), were treated to generate differentiated adipocytes as described [39,40]. These cells were used to confirm the ESR1-

mediated enhancer effect of 17ß-estradiol (E2) on the *Slc2a4* gene expression in an insulin sensitive cell. For that, differentiated adipocytes were cultivated without any treatment (control, C) or treated for 24 h with: 10 nM of water-soluble 17ß-estradiol (E2) (E4389, Sigma-Aldrich, St Louis, MO, USA); 10 nM of a selective ESR1 agonist PPT [1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole] (Sigma-Aldrich; St. Louis, MO, USA); 1 μM ESR1 antagonist MPP [1,3-bis(4-hydroxyphenyl)-4-methyl-5-(4-(2-piperidinylethoxy)phenol)-1H-pyrazoledihydrochloride] (Tocris, Avonmouth, Bristol, UK); and the combinations of E2+PPT or E2+MPP. At the end of the treatments, the cells were subjected to total RNA extraction for reverse transcription and quantitative polymerase chain reaction (RT-qPCR) analysis of *Slc2a4* mRNA, using *Gapdh* mRNA as internal control [40, 41].

Human embryonic kidney derived HEK293-FT cells, obtained from the American Type Culture Collection (ATCC®#CRL-3249, Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil), were used for transient transfection of *Slc2a4* constructs. The cells were allowed to proliferate in DMEM (Dulbecco's modified Eagle medium), 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin (Vitrocell/Embriolife, Campinas, SP, Brazil), in 75 cm^2 flasks, until reaching 90% confluence. Thus, cell samples were transferred to a 24-well plate, and allowed to reach 80% confluence. Thus, cells were transiently transfected with *Slc2a4* promoter fragments, carrying or not a mutation in the putative imperfect complete ERE, as detailed below in the luciferase assay section. After that, transfected cells were untreated or treated with E2, PPT and E2+PPT, under the same conditions described above for the 3T3-L1 adipocytes.

Luciferase Assay

Fragments of the 5'-flanking promoter of the mouse *Slc2a4* gene (*Slc2a4* transcript ENSMUST00000018710.12-GRCm38, https://www.ensembl.org, accessed on 12/2020), spanning the 5'-500, 5'-250 and 5'-149 regions, were PCR-amplified from mouse genomic DNA, using primers described in Supplementary Table S1. Restriction sites for Knpl and Xhol were incorporated at the 5'ends of the forward and reverse primers, respectively, to enable directional cloning upstream of the luciferase reporter gene in the pNL1.1[Nluc] vector (Promega, Madison, Wi, USA). Mutations in four bp of the putative ERE, located at positions -59/-46, in both fragments -500/-1 and -149/-1, were introduced using the primers described in Supplementary Table S1, and a mutagenesis kit (QuickChange XL Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA, USA), following suppliers' instructions. HEK293-FT cells were transiently transfected with either an empty Nanoluc vector or Nanoluc constructs of a wild-type or ERE-mutated *Slc2a4* promoter fragments, as described [38]. After transfection, cells were incubated for 8 h, in the presence of E2, PPT, or E2 + PPT, or without any drug, for further luciferase activity analysis, using the Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega, Madison, Wisconsin, USA). Luminescence was expressed as the Nanoluc/Firefly ratio.

Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM) of 4 to 7 samples, from at least two different batches of experiments, as specified in the legends. Comparisons among the groups were performed by one-way analysis of variance (ANOVA), followed by the

Tukey's multiple comparisons test, after confirming the normality of the data distribution by the Shapiro-Wilk test. Analyses were performed using GraphPad Prism (version 10.4.2).

RESULTS

ESR1 Mediates the Increase of *Slc2a4* mRNA Expression Induced by E2 in 3T3-L1 Adipocytes

As a proof of concept, we demonstrated that 3T3-L1 adipocytes cultivated for 24 h with E2 (E) or the selective ESR1 agonist PPT (P) increased (by 35%, $p<0.01$ vs. C) the *Slc2a4* mRNA expression (Figure 1a); besides, the treatment with the selective ESR1 antagonist MPP (M) treatment abolished ($p<0.01$ E+M vs E) the stimulating effect of E on *Slc2a4* mRNA expression (Figure 1b).

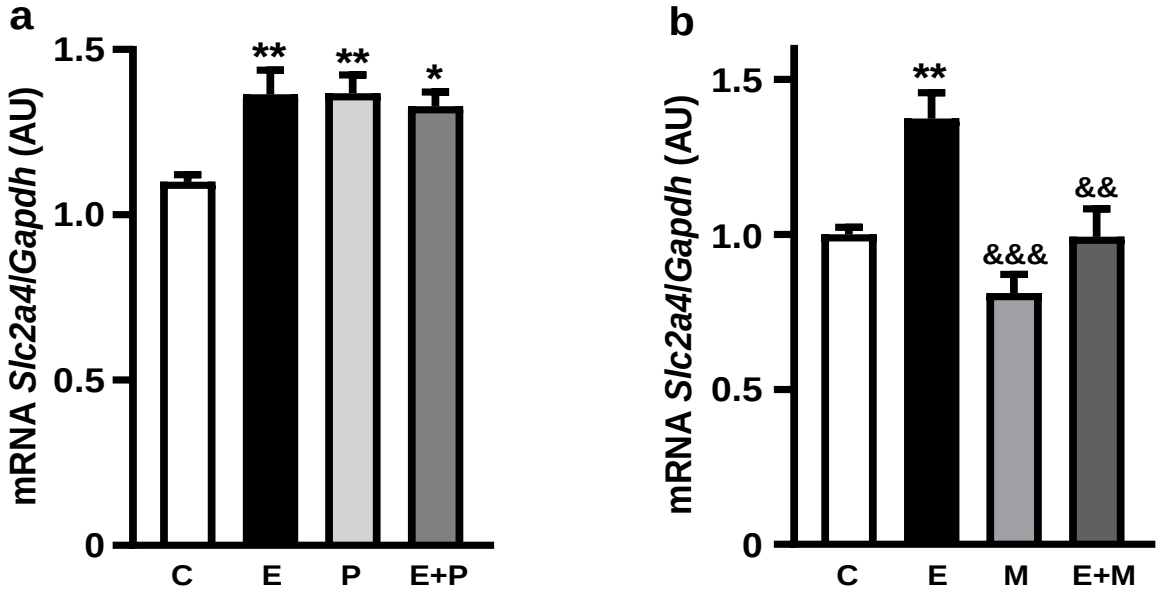

Figure 1. ESR1 mediates the increase of *Slc2a4*/GLUT4 expression induced by estrogen in 3T3-L1 adipocyte. *Slc2a4* mRNA (**A,D**) and GLUT4 protein (**B,C,E,F**) expression were analyzed in differentiates 3T3-L1 adipocytes cultivated for 24 h with 10 nM 17ß-estradiol (E), 10 nM ESR1 agonist PPT (1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole) (P) or 1 µM ESR1 antagonist MPP (1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(piperidinylethoxy)phenol]-1H-pyrazol dihydrochloride) (M), and compared with a control treatment without any drug. The *Slc2a4* mRNA was analyzed by RT-qPCR, using the *Gapdh* mRNA as a loading control; the GLUT4 protein was quantified in a total cellular membrane protein fraction, by Western blotting, using the Ponceau-stained membrane as a loading control. GLUT4 images representative of the results, with the respective Ponceau-stained membrane, are shown (**C**,**F**). Results were normalized considering the mean of C values as 1.0. Data are expressed as mean ± SEM of 5 (**B**) and 7 (**A**,**D**,**E**) samples, from at least 3 different batches of experiments. The means were compared by one-way ANOVA, followed by Tukey's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$ and $p < 0.001$ vs C; && $p < 0.01$ and &&& $p < 0.001$ vs E). AU, arbitrary units; C, control; E, 17ß-estradiol; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; GLUT4, solute carrier family 2, facilitated glucose transporter member 4; M, MPP (ESR1 antagonist); P, PPT (ESR1 agonist); *Slc2a4*, solute carrier family 2 member 4.

Progressive 5'-deletion of the -500/-1 *Slc2a4* Gene Promoter Modulates the Basal *Slc2a4* Transcriptional Activity

Firstly, we demonstrated that the nuclear ESR1 protein is spontaneous and abundantly expressed in HEK293-FT cells; however, its expression was modulated neither by E2 nor by PPT (Supplementary Figure S1).

a **Transcription factors acting on the 5'-500 bp of mouse *Slc2a4* gene promoter**

Protein	DNA Binding Site	Position	Reference
KLF15	CACCC	-486/-482	Gray, 2002 [34]
MYOD/HIF1A	CATTTGGC	-472/-465	Santalucia, 2001 [33]
MEF2	TAAAAATAGC	-446/-437	Liu, 1994 [31]
TR-alpha	GGGTTACTTCGGGGCA	-430/-415	Torrance, 1997 [32]
NR1H3	GGGTTACTTCGGGGCA	-430/-415	Dalen, 2003 [35]
CREB/CREM	TTACTTCG	-427/-420	Alves-Wagner, 2019 [38]
CEBPA	TTCAGAAAT	-275/-266	Kaestner, 1990 [30]
SP1	GGGGCGTGGCC	-141/-131	Im, 2006 [36]
NFKB	GGGCGTGGCC	-140/-131	Furuya, 2013 [37]
SREBP	CTGGGGTGTG	-127/-119	Im, 2006 [36]
NFKB	GGGCGTGTCT	-90 /-81	Furuya, 2013 [37]

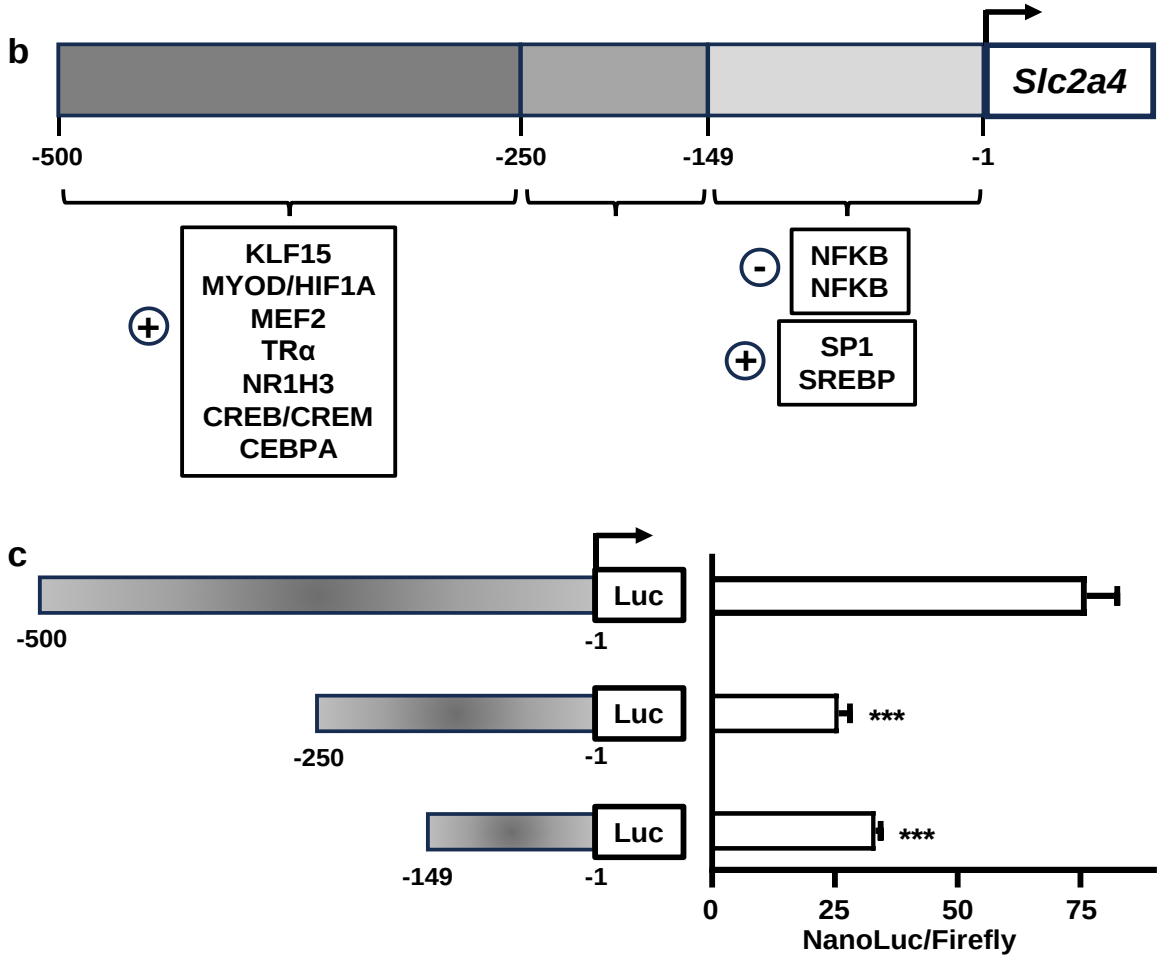

Figure 2. Progressive 5'-deletion of the 5'-500/-1 *Slc2a4* promoter modulates the basal *Slc2a4* tran-scriptional activity. **a**, Transcription factors acting on the 5'-500/-1 sequence of mouse *Slc2a4* gene with their respective DNA binding site sequence, segment position and reference. **b**, Schematic location of the binding sites for stimulatory (+) and inhibitory (-)

transcription factors encompassed in the -500/-250, -250/-149 and -149/-1 bp segments of the *Slc2a4* promoter. **c**, Basal *Slc2a4*-Luc promoter activity in HEK293-FT cells. Cells were transiently transfected with -500/-1, -250/-1 and -149/-1 *Slc2a4*-Luc reporter constructs (gray bars) and maintained for 8 h without any drug; after that, the respective basal *Slc2a4*-Luc reporter activity was measured and expressed as the ratio of NanoLuc to Firefly luminescence (white bars). Data are expressed as mean ± SEM of 4 samples, from at least two different batches of experiments. The means were compared by one-way ANOVA, followed by Tukey's multiple comparison test (*** $p < 0.001$ vs. -500/-1 construct). CEBPA, CCAAT/enhancer-binding protein alpha; CREB, cyclic AMP-responsive element-binding protein; CREM, cAMP-responsive element modulator; HIF1A: hypoxia-inducible factor 1-alpha; KLF15: Krueppel-like factor 15; MEF2: myocyte-specific enhancer factor; MYOD: myoblast determination protein; NFKB: nuclear factor NF-kappa-B; NR1H3: nuclear receptor subfamily 1 group H member 3 (former LXRα); SREBP: sterol regulatory element-binding protein; SP1: transcription factor Sp1; TRα: thyroid hormone receptor alpha.

Figure 2a shows several transcription factors characterized as modulators of the *Slc2a4* expression, with their respective DNA binding site sequence and segment position in the promoter. Figure 2b shows the schematic location of the binding sites of these transcription factors, with the indication of their stimulatory (+) or inhibitory (-) effect, in the three segments of the *Slc2a4* promoter analyzed. The complete segment (-500/-1 bp) encompasses eleven binding sites (for 9 enhancers and 2 inhibitors); the shortest segment (-149/-1 bp) encompasses three binding sites (for 2 inhibitors and 1 enhancer).

Figure 2c shows the basal *Slc2a4*-Luc activity of HEK293-FT cells transiently transfected with the three *Slc2a4*-Luc reporter constructs. Compared with the -500/-1 bp segment, the transcriptional activity (ratio of NanoLuc to Firefly luminescence) of the -250/-1 bp and of the -149/-1 bp decreased ($p<0.001$) by 66% and 56%, respectively. These results show that the loss of seven transcriptional activating sites of -500/-250 bp segment (Figures 2a,b) reduced the *Slc2a4* transcriptional activity by more than 50%.

The ESR1-mediated Estrogen-induced Enhancement of the *Slc2a4* Promoter Activity Involves the Imperfect Complete ERE Site Located at the -59/-46 Segment

Figure 3a shows the location of four short sequences similar to the first half-site of the consensus ERE, one short sequence similar to the second half-site of the consensus ERE, and a putative imperfect complete ERE site, all with their respective DNA sequences.

Figures 2b-d show the relative *Slc2a4*-Luc activity of the 5'-500 bp, 5'-250 bp and 5'-149 bp constructs, transfected into HEK293-FT cells, which were cultivated with E2 (E), PPT (P), the addition of E plus P (E+P) or maintained without any drug (C, control) for 8 hours. As compared with C cells, E, P and E+P similarly increased the *Slc2a4*-Luc activity of all three constructs by values from 130% to 190% ($p<0.05$ to $p<0.001$). For each construct, no difference was detected among the three treatments: E, P or E+P. Furthermore, the magnitude of the response to E, P and E+P, for the 5'-250 bp and for the 5'-149 bp, was not statistically different as compared by two-way ANOVA (comparison not shown in Figure 3).

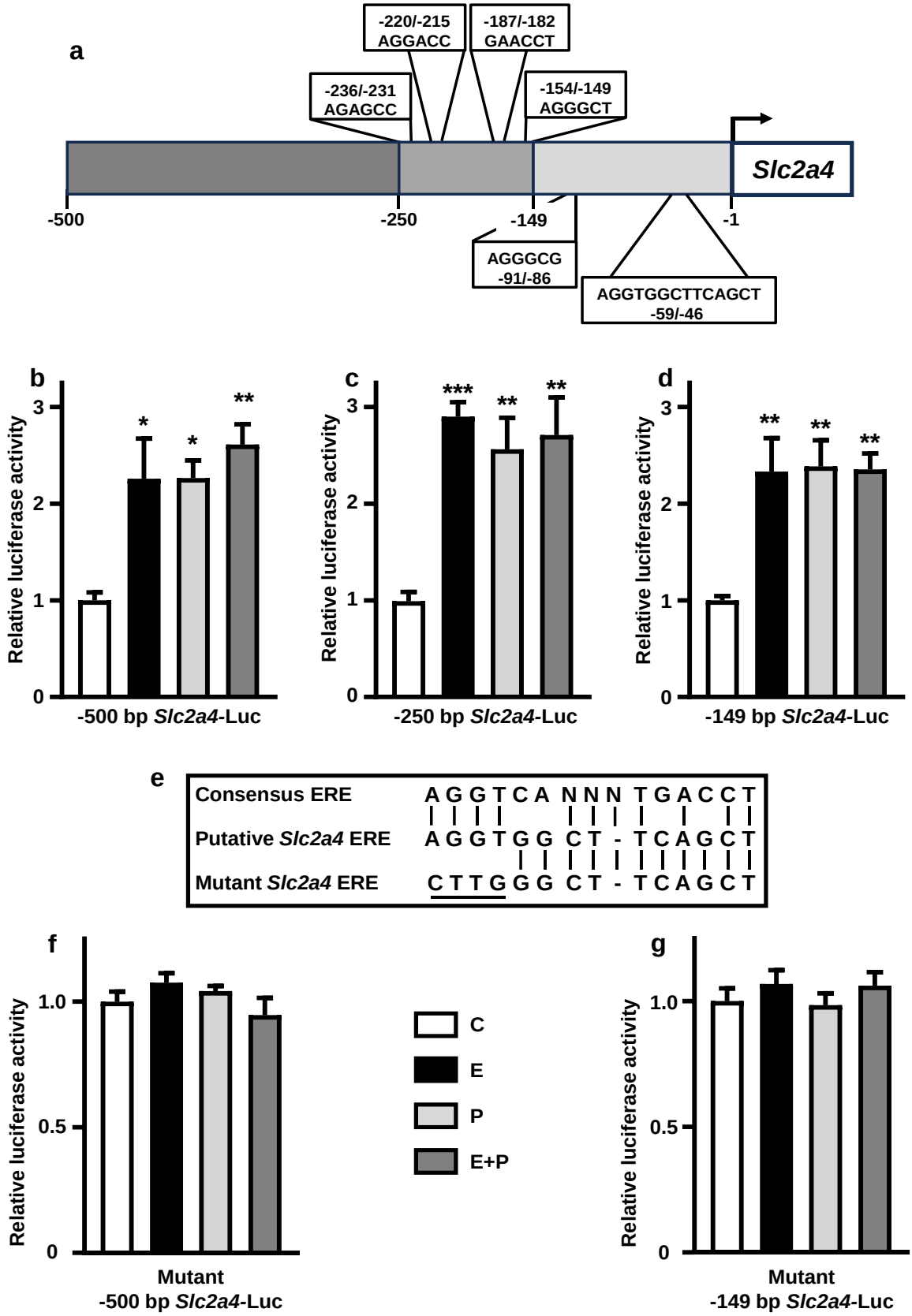

Figure 3. The ESR1-mediated estrogen-induced enhancement of *Slc2a4* promoter activity involves its binding into a putative imperfect complete estrogen responsive element (ERE) site located at the -59/-46 segment of the promoter. **a**, Localization in the -500/-1 sequence of mouse 5' *Slc2a4* promoter gene of four short sequences like to the first half-site of the

consensus ERE, one short sequence similar to the second half-site of the consensus ERE, and a putative imperfect complete ERE site, with their respective DNA binding sequences. **b,c,d**, ESR1 mediates the estrogen-induced expression of *Slc2a4* gene reporter. HEK293-FT cells were transiently transfected with the 5'-500 bp (**b**), 5'-250 bp (**c**) and 5'-149 bp (**d**) segments of *Slc2a4*-Luc constructs and then cultivated for 8 h with 10 nM 17β-estradiol (E), 10 nM ESR1 agonist PPT (1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole) (P), E plus P (E+P) or were maintained without any drug (C). Relative *Slc2a4*-Luc activities in each construct were normalized considering the mean of C values as 1.0. Data are expressed as mean ± SEM of 4 samples, from at least two different batches of experiments. The means were compared by one-way ANOVA, followed by Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. C). **e**, Comparison among the consensus ERE, the putative *Slc2a4* ERE, and a mutant *Slc2a4* ERE. The vertical lines indicate equal nucleotides between the sequences. In the mutant ERE sequence, four nucleotides (underlined) were replaced in the first half-site of the putative ERE. **f,g**, Relative *Slc2a4*-Luc activity of the ERE-mutated 5'-500 bp (**f**) and 5'-149 bp (**g**) segments does not increase in response to estrogen. HEK293-FT cells were transiently transfected with the 5'-500 bp or the 5'-149 bp *Slc2a4*-Luc con-structs, containing the mutant *Slc2a4* ERE and then cultivated for 8 h with 10 nM 17β-estradiol (E), 10 nM ESR1 agonist PPT (1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole) (P), E plus P (E+P) or were maintained without any drug (C). The transcriptional *Slc2a4*-Luc activity of the ERE-mutated segments was analyzed as described for panels B-D. Data are expressed as mean ± SEM of 6 samples, from at least two different batches of experiments. The means were compared by one-way ANOVA; no significant differences were observed among the groups. ERE, estrogen responsive element; ESR1, estrogen receptor 1 (former ER-α).

Figure 3e shows the comparison among the consensus complete ERE sequence, the putative imperfect ERE at the -59/-46 segment of the *Slc2a4* promoter, and the mutant sequence of the *Slc2a4* putative ERE, in which the first four nucleotides AGGT were replaced by CTTG as well. Vertical lines between the sequences indicate the same nucleotide.

Finally, Figures 3f,g show the relative *Slc2a4*-Luc activity of HEK293-FT cells transiently transfected with the 5'-500 bp or the 5'-149 bp *Slc2a4*-Luc constructs containing the mutant *Slc2a4* ERE. Compared with untreated cells (C), neither E nor P nor E+P had any effect in the *Slc2a4*-Luc activity, either in the 5'-500 bp or in the 5'-149 bp ERE-mutated *Slc2a4*-Luc constructs.

DISCUSSION

Estradiol (E2) exerts effects on the adipose tissue development, dyslipidemia, insulin resistance and inflammation, influencing the risk of developing obesity, metabolic syndrome and T2D [4,42-45]. Considering the estrogen pro-adipogenic effect [40], a hyperplasic development of adipose tissue is expected, thus improving glycemic homeostasis [4,44,46].

The complexity of the estrogen-dependent modulation of metabolic homeostasis may involve the estrogen receptors regulation of the *Slc2a4*/GLUT4, since estradiol may enhance or inhibit the *Slc2a4* gene, as it acts via ESR1 or ESR2, respectively [22-25,27,39,41]. Regarding the adipose tissue specifically, estrogen increases *Slc2a4*/GLUT4 expression [24, 41], because the adipocyte preponderantly expresses ESR1 [25]. Here, we

first confirmed the ESR1-mediated enhancer effect of E2 in the *Slc2a4* mRNA expression in adipocytes, by mimicking the E2 effect with the selective ESR1-agonist PPT, and abrogating the E2 effect with the selective ESR1-antagonist MPP, regulations that have already been proposed [27,39,41,47].

Concerning the *Slc2a4* gene expression regulation, we focused on the 5'-500 bp segment of the promoter, where most transcription factors bind [28], and putative imperfect ESR binding sites were proposed [27]. The basal *Slc2a4*-Luc reporter activity of HEK293-FT cells transfected with the constructs -500/-1 *Slc2a4*-Luc, -250/-1 *Slc2a4*-Luc and -149/01 *Slc2a4*-Luc reporter reveals that the loss of the 5'-500/-250 bp reduced more than 50% the transcriptional activity of the *Slc2a4*-Luc reporter, due to the presence of seven binding sites for important enhancer transcriptional factors in this segment.

To investigate the putative EREs of the *Slc2a4* gene in the transfected HEK293-FT cell, we first investigate the spontaneous expression of ESR1 in nuclear extract, which is abundant, but not modulated by E2 or PPT. That can be explained by the fact that this cell is derivative of the human embryonic kidney (HEK) 293 cell line, and the expression of ESR in embryonic kidney is well known [48].

As commented on above, the 5'-500 bp *Slc2a4* promoter contains five putative ERE half-site sequences, in which ESR1 might be regulating transcription as a monomer [15,17-21]. However, results show that the *Slc2a4*-Luc activity had been similarly increased either by E2 or PPT, in both the 5'-500 bp and the 5'-149 bp, and considering that from the -500 bp to the -149 bp segments four putative ERE half-sites were lost, it is tempting to speculate that ESR1 is not acting as a monomer in these ERE half-sites.

The most important result concerns the response of HEK293-FT cells transfected with the 5'-500 bp or 5'-149 bp ERE-mutated, in which neither E2 nor PPT increased in the *Slc2a4*-Luc activity, highlighting the relevance of the putative imperfect ERE site. These data led us to propose that the E2- and PPT-induced increase in *Slc2a4* transcriptional activity is an ESR1-mediated effect, involving the complete imperfect ERE (AGGTGGCTTCAGCT) located in the -59/-46 segment of the *Slc2a4* promoter. Furthermore, the complete absence of the enhancer effect of E2/PPT in the ERE-mutated 5'-500 bp reinforces that all half-sites encompassed in this segment are not being activated by ESR1 in a monomeric way in this cell.

ESR monomer is used to binding in ERE half-site, participating in cooperative regulation with other transcription factors [21, 27]. Regarding the *Slc2a4* gene expression in adipocytes, positive cooperative ESR1 regulation was proposed to occur in response to E2 and PPT with the transcription factor Sp1 (SP1) [47]. Although this cooperation might involve some ERE half-site, the fact that the mutated complete *Slc2a4* ERE completely lost the E2/PPT effect indicates that this cooperative regulation is not involving the direct ESR1 binding into a *Slc2a4* ERE half-site, even bearing in mind that these cells spontaneously express SP1 [49]. On the other hand, the ESR1/SP1 cooperative mechanism may also occur in a ligand-independent way, in which activated ESR1binds into SP1 and induces the SP1 direct binding into the DNA [18]. This ligand-independent mechanism is in agreement with previous study in 3T3L1 adipocytes reporting that PPT has increased the nuclear content of ESR1/SP1 complex as well as the SP1 binding into the *Slc2a4* promoter [47].

The proliferator-activated receptor gamma (PPARG) is a bizarre modulator of the *Slc2a4* expression, exerting repressive or stimulating effects, according to the ligand [50].

PPARG dimerizes with retinoid acid receptor RXR-alpha (RXRA), to bind into a consensus sequence presenting two repeats of AGGTCA (AGGTCANAGGTCA) [51]. Interestingly, the first half-site of the consensus ERE (AGGTCA) matches perfectly with the two half-sites of the PPARG/RXRA binding site, and ESR/PPARG/RXRA interactions have already been reported [51]. Despite the fact that PPARG-induced regulation of the *Slc2a4* gene (repressor) has been extensively investigated [50,51], to our knowledge, the exact PPARG/RXRA binding site into the *Slc2a4* gene is still unknown. A study related to this field described that PPARG/RXRA binds to the -66/+163 bp segment of the mouse *Slc2a4* gene, which includes a large transcriptional segment, without indicating its exact binding site [52]. Since *in silico* analysis indicates that there is no AGGTCA sequence in the 5'500 bp segment investigated here [27], the possible PPARG/ESR interaction was not analyzed in the present study.

CONCLUSIONS

This study reports the presence of one functional imperfect complete ERE AGGTGGCTTCAGCT in the *Slc2a4* gene, located at -59/-46 domain of the promoter region, which is important for the ESR1-mediate enhancing effect of 17ß-estradiol on *Slc2a4* mRNA expression. We hope that these results will be helpful in the future to establish some estrogen-related pharmacogenomic approach, capable of regulating GLUT4 abundance and ultimately contributing to improve glycemic homeostasis.

ABBREVIATIONS

Abbreviations (and names) of genes and proteins are in accordance with the HGNC and UniProt databases, respectively.

ANOVA: analysis of variance

AU: arbitrary units

CEBPA: CCAAT/enhancer-binding protein alpha

CREB: Cyclic AMP-responsive element-binding protein

CREM: cAMP-responsive element modulator

E2: 17ß-estradiol; ERE: estrogen responsive element

ESR: estrogen receptor protein

ESR1: estrogen receptor 1 protein (former ERα)

ESR1: *estrogen receptor 1* (human gene)

Esr1: *estrogen receptor 1* (mouse gene)

ESR2: estrogen receptor 2 protein (former ERß)

ESR2; *estrogen receptor 2* (human gene)

Gapdh: *glyceraldehyde-3-phosphate dehydrogenase* (mouse gene)

GLUT4: solute carrier family 2, facilitated glucose transporter member 4

HEK: human embryonic kidney

HIF1A: Hypoxia-inducible factor 1-alpha

IR: insulin resistance

Luc: luciferase reporter

M: MPP (ESR1 antagonist)

MEF2: Myocyte-specific enhancer factor

MPP: 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(piperidinylethoxy)phenol]-1H-pyrazol dihydrochloride

MYOD: Myoblast determination protein

NFKB: Nuclear factor NF-kappa-B

NR1H3: Nuclear receptor subfamily 1 group H member 3 (former LXRα)

P: PPT (ESR1 agonist)

PPT: 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole

RT-qPCR; reverse transcription and quantitative polymerase chain reaction

SEM: standard error of the mean

Slc2a4: solute carrier family 2 member 4 (mouse gene)

SREBP: Sterol regulatory element-binding protein

SP1: Transcription factor Sp1

T2D: type 2 diabetes

TRα: Thyroid hormone receptor alpha.

Author Contributions: U.F.M. and C.P.L. were responsible for the conception and design of the study. C.P.L. and K.C.R.G. carried out most of the experiments. M.M.O. contributed to the immunoblot and gene reporter assay; H.S.F. supervised the cell culture; C.N.A.B. contributed to execute the gene reporter assay; W.T.F. supervised the gene reporter analysis; U.F.M. was responsible for funding acquisition, writing and editing the manuscript; he also supervised the development of the study. All authors have read and agreed to the published version of the manuscript.

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REFERENCES

[1] Gessler, C.J.; Halsted, J.A.; Stetson, R.P. Effect of estrogenic substance on the blood sugar of female diabetics after the menopause. J. Clin. Investig. 1939, 18, 715–722.

[2] Rasio, E.; Antaki, A.; Campenhout, J. Diabetes Mellitus in Gonadal Dysgenesis: Studies of Insulin and Growth Hormone Secretion. Eur. J. Clin. Investig. 1976, 6, 59–66.

[3] Polychronakos, C.; Letarte, J.; Collu, R.; Ducharme, J. Carbohydrate intolerance in children and adolescents with Turner syndrome. J. Pediatr. 1980, 96, 1009–1014.

[4] Carr, M.C. The emergence of the metabolic syndrome with menopause. J. Clin. Endocrinol. Metab. 2003, 88, 2404–2411.

[5] Dunaif, A.; Segal, K.R.; Futterweit, W.; Dobrjansky, A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. Diabetes 1989, 38, 1165–1174.

[6] Livingstone, C.; Collison, M. Sex steroids and insulin resistance. Clin. Sci. 2002, 102, 151–166.

[7] Lopez, L.M.; Grimes, D.A.; Schulz, K.F. Steroidal contraceptives: Effect on carbohydrate metabolism in women without diabetes mellitus. Cochrane Database Syst. Rev. 2014, 2019, 31711271

[8] Smith, E.P.; Boyd, J.; Frank, G.R.; Takahashi, H.; Cohen, R.M.; Specker, B.;Williams, T.C.; Lubahn, D.B.; Korach, K.S. Estrogen Resistance Caused by a Mutation in the Estrogen-Receptor Gene in a Man. N. Engl. J. Med. 1994, 331, 1056–1061.

[9] Faustini-Fustini, M.; Rochira, V.; Carani, C. Oestrogen deficiency in men: Where are we today? Eur. J. Endocrinol. 1999, 140, 111–129.

[10] Elbers, J.M.H.; Giltay, E.J.; Teerlink, T.; Scheffer, P.G.; Asscheman, H.; Seidell, J.C.; Gooren, L.J.G. Effects of sex steroids on components of the insulin resistance syndrome in transsexual subjects. Clin. Endocrinol. 2003, 58, 562–571.

[11] Belgorosky, A.; Guercio, G.; Pepe, C.; Saraco, N.; Rivarola, M. Genetic and Clinical Spectrum of Aromatase Deficiency in Infancy, Childhood and Adolescence. Horm. Res. Paediatr. 2009, 72, 321–330.

[12] Mueckler, M.; Thorens, B. The SLC2 (GLUT) family of membrane transporters. Mol. Asp. Med. 2013, 34, 121–138.

[13] Klip, A.; McGraw, T.E.; James, D.E. Thirty sweet years of GLUT4. J. Biol. Chem. 2019, 294, 11369–11381.

[14] Machado, U.F.; Shimizu, I.; Saito, M. Reduced content and preserved translocation of glucose transporter (GLUT 4) in white adipose tissue of obese mice. Physiol. Behav. 1994, 55, 621–625.

[15] Nilsson, S.; Mäkelä, S.; Treuter, E.; Tujague, M.; Thomsen, J.; Andersson, G.; Enmark, E.; Pettersson, K.;Warner, M.; Gustafsson, J.-Å. Mechanisms of Estrogen Action. Physiol. Rev. 2001, 81, 1535–1565.

[16] Heldring, N.; Pike, A.; Andersson, S.; Matthews, J.; Cheng, G.; Hartman, J.; Tujague, M.; Ström, A.; Treuter, E.; Warner, M.; Gustafsson J.-A. Estrogen Receptors: How Do They Signal and What Are Their Targets. Physiol. Rev. 2007, 87, 905–931.

[17] Eriksson, M.A.L.; Nilsson, L. Structural and dynamic differences of the estrogen receptor DNA-binding domain, binding as a dimer and as a monomer to DNA: Molecular dynamics simulation studies. Eur. Biophys. J. 1999, 28, 102–111.

[18] Safe, S. Transcriptional activation of genes by 17ß-estradiol through estrogen receptor-Sp1 interactions. Vitam. Horm. 2001, 62, 231–252.

[19] Hewitt, S.C.; Li, Y.; Li, L.; Korach, K.S. Estrogen-mediated Regulation of Igf1 Transcription and Uterine Growth Involves Direct Binding of Estrogen Receptor α to Estrogen-responsive Elements. J. Biol. Chem. 2009, 285, 2676–2685.

[20] Liu, D.; Deschamps, A.; Korach, K.S.; Murphy, E. Estrogen-Enhanced Gene Expression of Lipoprotein Lipase in Heart Is Antagonized by Progesterone. Endocrinology, 2007, 149, 711–716.

[21] Safe, S.; Kim, K. Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. J. Mol. Endocrinol. 2008, 41, 263–275.

[22] Barros, R.P.A.; Machado, U.F.; Warner, M.N.; Gustafsson, J.-Å. Muscle GLUT4 regulation by estrogen receptors ERß and ERα. Proc. Natl. Acad. Sci. USA. 2006, 103, 1605–1608.

[23] Barros, R.P.; Machado, U.F.; Gustafsson, J.-Å. Estrogen receptors: New players in diabetes mellitus. Trends Mol. Med. 2006, 12, 425–431.

[24] Barros, R.P.A.; Gabbi, C.; Morani, A.; Warner, M.; Gustafsson, J.-Å. Participation of ERα and ERß in glucose homeostasis in skeletal muscle and white adipose tissue. AJP Endocrinol. Metab. 2009, 297, E124–E133.

[25] Barros, R.P.; Gustafsson, J.-Å. Estrogen Receptors and the Metabolic Network. Cell. Metab. 2011, 14, 289–299.

[26] Giannella, M.L.C.C.; Machado, U.F. SLC2A4gene: A promising target for pharmacogenomics of insulin resistance. Pharmacogenomics 2013, 14, 847–850.

[27] Gregorio, K.C.R.; Laurindo, C.P.; Machado, U.F. Estrogen and Glycemic Homeostasis: The Fundamental Role of Nuclear Estrogen Receptors ESR1/ESR2 in Glucose Transporter GLUT4 Regulation. Cells., 2021; 10, 99.

[28] Im, S-S.; Kwon, S.-K.; Kim, T.-H.; Kim, H.; Ahn, Y.-H. Regulation of glucose transporter type 4 isoform gene expression in muscle and adipocytes. IUBMB Life 2007, 59, 134–145.

[29] Karnieli, E.; Armoni, M. Transcriptional regulation of the insulin-responsive glucose transporter GLUT4 gene: From physiology to pathology. Am. J. Physiol. Metab. 2008, 295, E38–E45.

[30] Kaestner, K.H.; Christy, R.J.; Lane, M.D. Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. Proc. Natl. Acad. Sci. USA. 1990, 87, 251-5.

[31] Liu, M.L.; Olson, A.L.; Edgington, N.P.; Moye-Rowley, W.S.; Pessin, J.E. Myocyte enhancer factor 2 (MEF2) binding site is essential for C2C12 myotube-specific expression of the rat GLUT4/muscle-adipose facilitative glucose transporter gene. J. Biol. Chem. 1994, 269, 28514-21.

[32] Torrance, C.J.; Usala, S.J.; Pessin, J.E.; Dohm, G.L. Characterization of a low affinity thyroid hormone receptor binding site within the rat GLUT4 gene promoter. Endocrinology 1997, 138, 1215-23.

[33] Santalucía, T.; Moreno, H.; Palacín, M.; Yacoub, M.H.; Brand, N.J.; Zorzano, A. A novel functional co-operation between MyoD, MEF2 and TRalpha1 is sufficient for the induction of GLUT4 gene transcription. J Mol Biol. 2001, 314, 195-204.

[34] Gray, S.; Feinberg, M.W.; Hull, S.; Kuo, C.T.; Watanabe, M.; Sen-Banerjee, S.; DePina, A; Haspel, R.; Jain, M.K. The Krüppel-like factor KLF15 regulates the insulin-sensitive glucose transporter GLUT4. J. Biol. Chem. 2002, 277, 34322-8.

[35] Dalen, K.T.; Ulven S.M.; Bamberg, K.; Gustafsson, J-A.; Nebb, H.I. Expression of the insulin-responsive glucose transporter GLUT4 in adipocytes is dependent on liver X receptor alpha. J. Biol. Chem. 2003, 28, 48283-48291.

[36] Im, S.-S.; Kwon, S.-K.; Kang, S.-Y.; Kim, T.-H.; Kim, H.-I.; Hur, M.-W.; Kim, K.-S.; Ahn, Y.-H. Regulation of GLUT4 gene expression by SREBP-1c in adipocytes. Biochem. J. 2006, 399, 131-9.

[37] Furuya, D.T.; Neri, E.A.; Poletto, A.C.; Anhê, G.F.; Freitas, H.S.; Campello, R.S.; Rebouças, N.A.; Machado, U.F. Identification of nuclear factor-kappaB sites in the Slc2a4 gene promoter. Mol. Cell. Endocrinol. 2013, 370, 87–95.

[38] Alves-Wagner, A.B.; Yonamine, C.Y.; Fatima, L.A.; Festuccia, W.; Machado, F.M. Sympathetic regulation of Slc2a4 gene expression: Participation of a putative cAMP responsive element (CRE) site in the Slc2a4 promoter. Cell Physiol Biochem. 2019, 52, 580-594.

[39] Campello, R.S.; Fatima, L.A.; Barreto-Andrade, J.N.; Lucas, T.F.; Mori, R.C.; Porto, C.S.; Machado, U.F. Estradiol-induced regulation of GLUT4 in 3T3-L1 cells: Involvement of ESR1 and AKT activation. J. Mol. Endocrinol. 2017, 59, 257–268.

[40] Fatima, L.A.; Campello, R.S.; Barreto-Andrade, J.N.; Passarelli, M.; Santos, R.S.; Clegg DJ.; Machado, U.F. Estradiol stimulates adipogenesis and Slc2a4/GLUT4 expression via ESR1-mediated activation of CEBPA. Mol. Cell. Endocrinol. 2019, 498, 110447.

[41] Campello, R.S.; Alves-Wagner, A.B.; Lucas, T.F.; Mori, R.C.; Furuya, D.T.; Porto, C.S.; Machado, U.F. Estrogen receptor 1 agonist PPT stimulates Slc2a4 gene expression and improves insulin-induced glucose uptake in adipocytes. Curr. Top. Med. Chem. 2012, 12, 2059–2069.

[42] Pfeilschifter, J.; Köditz, R.; Pfohl, M.; Schatz, H. Changes in proinflammatory cytokine activity after menopause. Endocr. Rev. 2002, 23, 90–119.

[43] Tchernof, A.; Desmeules, A.; Richard, C.; Laberge, P.; Daris, M.; Mailloux, J.; et al., Ovarian hormone status and abdominal visceral adipose tissue metabolism. J. Clin. Endocrinol. Metab. 2004, 89, 3425–3430.

[44] Meyer, M.R.; Clegg, D.J.; Prossnitz, E.R.; Barton, M. Obesity, insulin resistance and diabetes: Sex differences and role of oestrogen receptors. Acta Physiol. 2010, 203, 259–269.

[45] Stubbins, R.E.; Holcomb, V.B.; Hong, J.; Núñez, N.P. Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance. Eur. J. Nutr. 2012, 51, 861–870.

[46] Gustafson, B.; Hedjazifar, S.; Gogg, S.; Hammarstedt, A.; Smith, U. Insulin resistance and impaired adipogenesis. Trends Endocrinol. Metabol. 2015, 26, 193–200.

[47] Barreto-Andrade, J.N.; De Fátima, L.A.; Campello, R.S.; Guedes, J.A.C.; De Freitas, H.S.; Machado, M.M.O.U.F. Estrogen Receptor 1(ESR1) Enhances Slc2a4/GLUT4 Expression by a SP1 Cooperative Mechanism. Int. J. Med. Sci. 2018, 15, 1320–1328.

[48] Torre, S.D.; Rando, G.; Meda, C.; Ciana, P.; Ottobrini, L.; Maggi, A. Transcriptional activity of oestrogen receptors in the course of embryo development. J. Endocrinol. 2018, 238, 165-176.

[49] O'Connor, L.; Gilmour, J.; Bonifer, C. The Role of the Ubiquitously Expressed Transcription Factor Sp1 in Tissue-specific Transcriptional Regulation and in Disease. Yale J. Biol. Med. 2016, 89, 513-525.

[50] Armoni, M.; Harel, C.; Karnieli, E. Transcriptional regulation of the GLUT4 gene: From PPAR- and FOXO1 to FFA and inflammation. Trends Endocrinol. Metab. 2007, 18, 100–107.

[51] Chandra, V.; Huang, P.; Hamuro, Y.; Raghuram, S.; Wang, Y.; Burris, T.P.; Rastinejad, F. Structure of the intact PPAR—RXR nuclear receptor complex on DNA. Nature. 2008, 456, 350–356.

[52] Armoni, M.; Kritz, N.; Harel, C.; Bar-Yoseph, F.; Chen, H.; Quon, M.J.; Karnieli, E. Peroxisome Proliferator-activated Receptor Represses GLUT4 Promoter Activity in Primary Adipocytes, and Rosiglitazone Alleviates This Effect. J. Biol. Chem. 2003, 278, 30614–30623.

Supplementary Table S1

Table S1. Sequences of primers used to obtain the 5'-500, 5'-250 and 5'-149 bp segments of the mouse *Slc2a4* gene, and to acquire a putative ERE-mutated domain. In lowercase letters, the sites for restriction enzymes; in bold letters, the mutated nucleotides.

Primer	Forward	Reverse
-500/-1	gcggtaccGGGACAGGCTGGGACACCCGG	gactcgagTCTCCGGGATCTAGTGAGACC
-250/-1	gcggtaccTTTGGATGGCGGGAAGAGCCT	gactcgagTCTCCGGGATCTAGTGAGACC
-149/-1	gcggtaccAGGGGTGGGGGCGTGGCCTTT	gactcgagTCTCCGGGATCTAGTGAGACC
Mutant	GCGGAGAGCTGAAGCC**CAAG**CCCCACTCC CGCCCCG	CGGGGCGGGAGTGGGG**CTTG**GGCTTCAGC TCTCCGC

ERE, estrogen responsive element; *Slc2a4*: *solute carrier family 2 member 4* (mouse gene).

Supplementary Figure S1

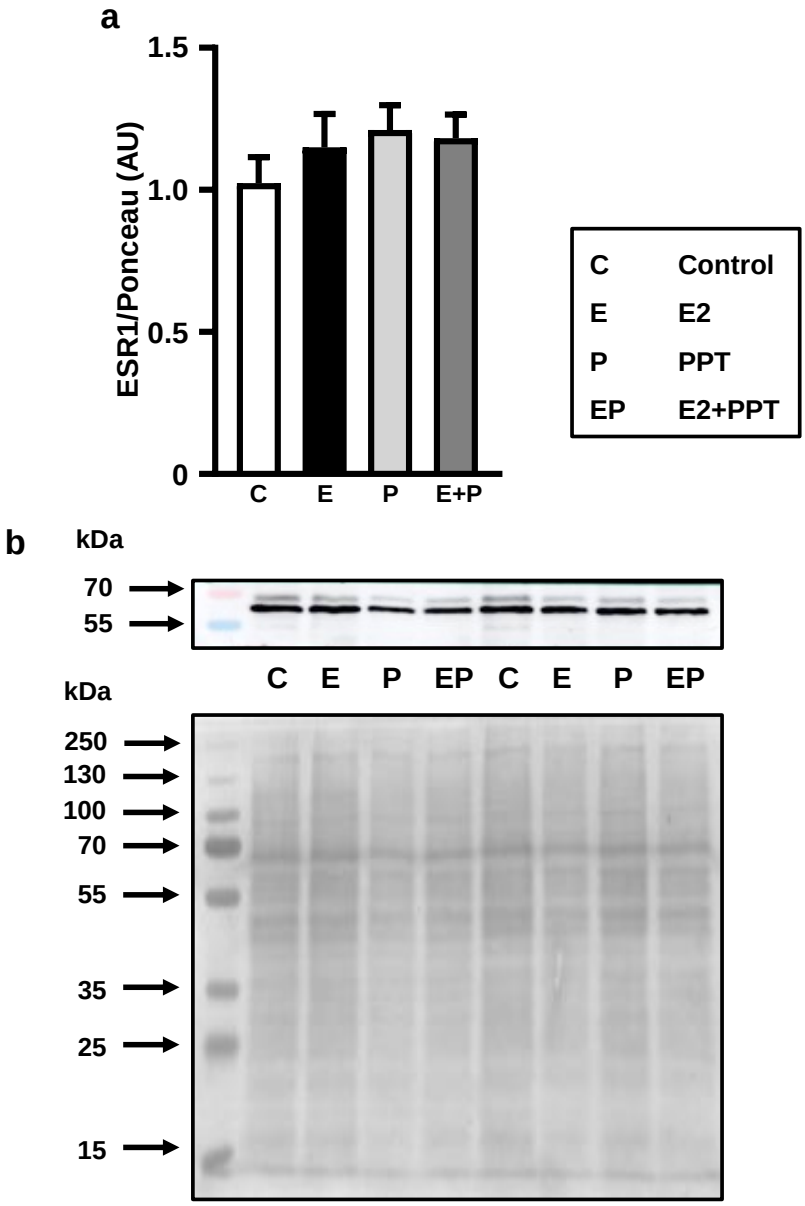

Figure S1. *HEK 293FT cells express ESR1, but that is not modulate by E2 or PPT.* ESR1 protein expression was analyzed in HEK 293FT cells cultivated for 24 h with 10 nM 17β-estradiol (E), 10 nM ESR1 agonist PPT (1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole) (P) or E+P, and compared with a control treatment without any drug. The ESR1 protein was quantified in a nuclear protein extract, by Western blotting, using the Ponceau-stained membrane as a loading control. Results were normalized considering the mean of C values as 1.0. **A**, Data expressed as mean ± SEM of 6 samples, from at least 3 different batches of experiments. The means were compared by one-way ANOVA, followed by Tukey's multiple comparison test (** $p < 0.01$, *** $p < 0.001$ vs. C; &&& $p < 0.01$ vs E; # $p < 0.05$ vs M). **B**, Images of one immunoblotting, with the respective Ponceau-stained membrane used for normalization. AU, arbitrary units; C, control; E, 17β-estradiol; ESR1, estrogen receptor 1 (former ER-α); M, MPP; P, PPT.