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**Insulin resistance of pregnancy involves  
estrogen-induced repression of muscle GLUT4**

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**Keywords:** Beta-estradiol, ER $\alpha$ , ER $\beta$ , GLUT4 expression, skeletal muscle, L6 cell, gestational diabetes, insulin resistance

# **Abstract**

Pregnancy is accompanied by hyperestrogenism, however, the role of estrogens in the gestational-induced insulin resistance is unknown. Skeletal muscle plays a fundamental role in this resistance, where GLUT4 regulates glucose uptake. We investigated: 1) effects of oophorectomy and estradiol (E2) on insulin sensitivity and GLUT4 expression. E2 (~200 nM) for 7 days decreased sensitivity, reducing ~30% GLUT4 mRNA and protein ( $P<0.05$ ) and plasma membrane expression in muscle; 2) the expression of ER $\alpha$  and ER $\beta$  in L6 myotubes, showing that both coexpress in the same nucleus; 3) effects of E2 on GLUT4 in L6, showing a time- and dose-dependent response. High concentration (100 nM) for 6 days reduced ~25% GLUT4 mRNA and protein ( $P<0.05$ ). Concluding, E2 regulates GLUT4 in muscle, and at high concentrations, such as in pregnancy, reduces GLUT4 expression and, in vivo, decreases insulin sensitivity. Thus, hyperestrogenism may be involved in the pregnancy-induced insulin resistance and/or gestational diabetes.

## Introduction

During progression of normal pregnancies, insulin sensitivity decreases, and insulin resistance arises (Reece *et al.*, 1994) followed or not by diabetes (Kaaaja and Greer, 2005). Gestational diabetes mellitus (GDM) has been observed in 2 to 5% of the pregnancies, and its prevalence has increased in the last decade (Shaata and Groop, 2007). Besides, persistent or recurrent impaired glucose homeostasis after gestational diabetes is frequent (Case *et al.*, 2006, Haynes *et al.*, 2003).

Although genetic and behavioral risk factors have been related to GDM, changes in the hormonal environment of pregnant females, which decrease insulin sensitivity, seem to be involved in the development of diabetes (Kaaaja and Greer, 2005, Okuno *et al.*, 1995). In addition to some placental hormones that decrease insulin sensitivity, high estrogen concentration could be involved in the gestational-induced insulin resistance, since other conditions characterized by elevated levels of estrogen are also accompanied by insulin resistance (Solomon *et al.*, 2001, Okuno *et al.*, 1995, Livingstone and Collison, 2002, Kaaaja and Greer, 2005, Dunaif *et al.*, 1989).

Redundant control mechanisms operate to maintain glucose homeostasis; however, insulin is the single most important hormone, regulating carbohydrate, protein and fat metabolism muscle, adipose tissue and liver (Eckel *et al.*, 2005). In muscle and adipose tissues the insulin-sensitive glucose transporter GLUT4 is predominantly expressed (Gould and Holman, 1993), and is the rate-limiting step of the insulin-induced glucose uptake (Rea and James, 1997). Insulin, after binding into its receptor, activates an intracellular pathway that culminates with the translocation of GLUT4-containing vesicles into the plasma membrane (Zhou *et al.*, 1999), and then, glucose uptake acutely increases in these tissues.

Recently, we have reported that selective estrogen receptor (ER) alpha (ER $\alpha$ ) and beta (ER $\beta$ ) knockout mice showed increased and reduced GLUT4 protein; respectively (Barros *et al.*, 2006b). This suggests that ER $\alpha$ - and ER $\beta$ -activity could have different effects on the glucose uptake, thus indicating that estradiol participates in glycemic homeostasis. In fact, the correlation between increased E2 and reduced adipose tissue GLUT4 content has already been described in insulin resistant conditions such as normal pregnancy, GDM or Polycystic Ovarian Syndrome (Garvey *et al.*, 1993, Okuno *et al.*, 1995, Rosenbaum *et al.*, 1993). However, in skeletal muscle, the most important site of insulin resistance (Zorzano *et al.*, 1998, Ryder *et al.*, 2001), the GLUT4 expression in pregnant subjects has not been reported yet. Only one study investigated the E2 effect on muscle GLUT4 of oophorectomized rats treated or not with E2, showing that physiological levels of E2 decreased GLUT4 protein in red quadriceps whereas had no effect on white quadriceps (Campbell and Febbraio, 2002).

Based on that, we hypothesized that high estrogen levels could repress GLUT4 expression in muscle, thus participating in the gestational-induced insulin resistance. Therefore, the aim of this study was to investigate: 1) the in vivo effect of E2 treatment on the expression of GLUT4 gene in muscle and adipose tissue of oophorectomized rats; 2) the expression and colocalization of ER $\alpha$  and ER $\beta$  on L6 myotubes in order to confirm that these cells are target of estrogenic action; and 3) the in vitro effects of E2 on GLUT4 expression in L6 myotubes. Furthermore, by providing in vivo and in vitro E2 concentrations similar to those observed in pregnancy, we expect to reveal mechanisms involved in the gestational insulin resistance.

For the first time, we report that oophorectomy increased GLUT4 mRNA and protein in skeletal muscle, which is reversed by the E2 treatment for 7 days. We also show that L6 myotubes express both  $\alpha$  and  $\beta$  isoforms of ER, and the treatment of these cells for 6 days with high E2 levels decreased the GLUT4 mRNA and protein expression. These results together showed that a high concentration of estrogen, such as that observed in pregnancy, suppresses the GLUT4 gene expression, which may participate in the gestational-induced insulin resistance.

### Materials and Methods

All experiments were in accordance with the guidelines of the Brazilian College of Animal Experimentation (COBEA), and were approved by the Ethical Committee for Animal Research (CEEAA) of the Institute of Biomedical Sciences, University of São Paulo (protocol n° 060/2000).

#### *Animals:*

Two-month-old female Wistar rats were housed in the animal facility of the Department of Physiology in a controlled environment of 12-h light/12-h dark, and fed standard pellet diet and tap water ad libitum. After two regular cycles, the animals were subjected to oophorectomy (Ox) or to sham-operation (C). Surgeries were performed in sodium pentobarbital (35 mg/Kg BW, *i.p.*) anesthetized rats; ovaries were assessed by bilateral posterior incisions, and removed in ooforectomized rats or only manipulated in sham-operated rats. Fourteen days after the surgical procedures, oophorectomized rats were subcutaneously treated with sunflower oil (Ox-O) or E2 (Ox-E) (50 $\mu$ g/day) for 3 or 7 days. Control and untreated Ox rats were investigated 19 to 21 days after the day of the surgeries. Control rats were studied in proestrus phase, after confirming two regular cycles.

On the day of the experiment, animals were anesthetized with sodium pentobarbital (35 mg/Kg BW, *i.p.*). Blood was collected from the inferior *vena cava* for further analysis

of plasma  $17\beta$ -estradiol (RIE, Coat-a-Count, Diagnostic Products, Los Angeles, CA, USA), insulin (RIE, Coat-a-Count, Diagnostic Products, Los Angeles, CA, USA) and glucose (Glucose-Oxidase, Analisa Diagnostica, Sao Paulo, Brazil). Values of plasma glucose (mg/dL) and insulin ( $\mu$ U/mL) were used to calculate the glucose/insulin (G/I) index, as a parameter of insulin sensitivity of non-fasted animals (de Carvalho Papa *et al.*, 2002). The experiments were performed from 10:00 to 12:00 h, after 4 to 6 hours of food deprivation. Periuterine white adipose tissue (WAT) and *gastrocnemius* skeletal muscle (SM) were removed, weighted and stored at -70 °C for further GLUT4 protein and mRNA analysis.

#### Cell culture:

L6 myoblasts obtained from ATCC (CRL 1458) were propagated in flasks (Corning, 75cm<sup>2</sup> TC-treated) containing Dulbecco's Modified Eagle's Medium (DMEM - Gibco, Invitrogen) and 10% fetal bovine serum (FBS - Gibco, Invitrogen) in a Forma Scientifica incubator at 37°C and 5% CO<sub>2</sub>. When cells reached 90% confluence, the cells were transferred to either cell culture dishes (Corning, 100mm TC-treated), or to a chamber slide system (Nunc, Lab-Tek II CC2 Chamber Slide System). The cells were allowed to differentiate into myotubes by changing the medium to DMEM, 2% FBS for 6 days (Yonemitsu *et al.*, 2001), and then used for the evaluation of GLUT4 expression or ERs expression and colocalization.

After differentiation, the medium was changed to DMEM Phenol-Red Free (Gibco, Invitrogen) without FBS for 24 hours, to prevent any estrogenic action (Berthois *et al.*, 1986). The cells were then treated in the same medium containing 2% dextran-treated FBS (Sigma, C-6241) (Nogueira and Brentani, 1996) for 1, 2, 3 and 6 days, under different concentrations (0.1 to 100 nM) of water soluble estradiol (Sigma, E-4389).



*Detection and colocalization of ER $\alpha$  and ER $\beta$  in L6 myotubes by immunofluorescence:*

After the differentiation period, slides containing the myotubes were washed in PBS and dried at room temperature for 20 minutes, followed by fixation in 4% PFA for 20 minutes, at 4°C. Following PBS washing, cells were incubated in PBS/ 1% Triton X-100 for 20 minutes at room temperature. To block the nonspecific binding, sections were incubated in 3% BSA for 1 h at 4°C, followed by PBS washing. Sections were then incubated with both anti-ER $\alpha$  (Santa Cruz Biotechnology, MC-20) and anti-ER $\beta$  [produced and donate by Prof. Jan-Åke Gustafsson, Karolinska Institute, Department of Biosciences and Nutrition, Stockholm, Sweden (Saji *et al.*, 2000)] at a dilution of 1:200, in 1% BSA and 0.1% P40 in PBS overnight at 4°C. BSA replaced primary antibodies in negative controls. After PBS washing, sections were incubated with the corresponding secondary antibodies (FITC anti-rabbit and Cy3 anti-chicken, Jackson Immunoresearch) at a dilution of 1:200 in PBS for 2h at room temperature. The sections were washed in PBS and lightly counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and directly mounted in Vectashield antifading medium (Vector Laboratories). The sections were examined under a Zeiss fluorescence microscope with filters suitable for selectively detecting the fluorescence of FITC (green), Cy3 (red) and DAPI (blue). For colocalization, images from the same section but showing different antigen signals were overlaid. Cells where both FITC- and Cy3-conjugated secondary antibodies were present have an orange or yellow color.

*GLUT4 mRNA analysis:*

Total RNA was extracted from frozen tissue (0.1g for skeletal muscle and 0.6g for adipose tissue) or fresh myotubes using Trizol<sup>®</sup> reagent (Gibco BRL, Life Technologies,

Inc., Gaithersburg, USA) according to the manufacturer's instructions. Total RNA (20  $\mu$ g) was electrophoresed in 1.3% formaldehyde-agarose gel, blotted, cross-linked onto nylon membrane and then hybridized with rat GLUT4 cDNA probe. The membrane was washed and exposed to autoradiography. Later, the membrane was stripped and hybridized with  $\beta$ -actin cDNA probe. Blots were analyzed by scanner densitometry, using the Image Master1D software (Pharmacia Biotech, Upsalla, SW). The results obtained from the GLUT4 mRNA blots were related to the respective  $\beta$ -actin values and normalized considering the mean of control values (tissues study) or zero E2 values (cells study) of each gel as 100. Results were expressed as arbitrary units (AU).

*GLUT4 protein analysis:*

GLUT4 protein was assessed in a total cellular membrane fraction, as previously described (Machado et al., 1993, de Carvalho Papa et al., 2002). Briefly, the white adipose tissue was homogenized using a Polytron (Brinkmann Instruments, Westbury, NY) in 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 250 mmol/L sucrose buffer, pH 7.4, and centrifuged at 3,000 x g for 15 min. The fat cake was discarded, and the supernatant, a fat free extract was recovered as a total cellular membrane fraction. Skeletal muscle was homogenized in the same buffer, and centrifuged at 1,000 x g for 10 min. The supernatant was kept; the pellet was resuspended in 1/3 of the initial volume of the buffer, and centrifuged again at 1,000 x g for 10 min. Both supernatants were mixed and subjected to a 150,000 x g centrifugation for 75 min. The final pellet was resuspended as a total cellular membrane fraction in 1 mL of buffer. L6 myotubes were homogenized in a polytron in Tris Hcl 10 mM, EDTA 1 mM, sucrose 250 mM buffer containing protease inhibitors (aprotinine 15  $\mu$ g/ml, leupeptine 5  $\mu$ g/mL e PMSF 0.07  $\mu$ g/mL) and centrifuged at 1,000 x g for 10 min, at

170 4°C. The supernatant was recovered and again centrifuged at a 100,000 x g for 75 min at  
171 4°C. The final pellet was resuspended in the same buffer, and contained both plasma  
172 membrane and microsomal fractions. Subcellular membrane fractionation was performed  
173 in tissue samples homogenized as described above. Muscle samples were submitted to a  
174 series of differential centrifugations (Mitsumoto and Klip, 1992). The crude homogenate  
175 was centrifuged at 760 x g, 10 minutes, 4 °C. Supernatant was centrifuged at 31,000 x g for  
176 60 minutes, and the pellet was resuspended in homogenization buffer as the plasma  
177 membrane rich fraction (PM). Microsomal fraction (M) was obtained after supernatant  
178 ultra centrifugation at 190,000 x g. WAT samples were processed differently (Machado *et*  
179 *al.*, 1994). The fat-free extract of the 3,000 x g centrifugation was subsequently centrifuged  
180 at 12,000 x g for 15 minutes. The pellet was resuspended as the PM fraction and the  
181 supernatant was centrifuged at 28,000 x g for 15 minutes. The pellet was discarded and the  
182 supernatant was centrifuged at 146,000 x g for 75 minutes. The final pellet was  
183 resuspended as the M fraction. All procedures were performed at 4°C. Total protein  
184 concentration of the samples was evaluated by Lowry's method (Lowry *et al.*, 1951).  
185 GLUT4 protein was assessed by Western blotting method, with anti-GLUT4 antiserum  
186 (Rabbit Polyclonal Anti-GLUT4, AB1346, Chemicon International Inc., Temecula, CA,  
187 USA), and submitted to enhanced chemiluminescent detection (ECL™ Western Blotting  
188 Detection, RPN2106, Amersham Pharmacia Biotech, Amersham, UK). Autoradiograms  
189 were analyzed as described for Northern blotting. Results of GLUT4 blots were  
190 normalized considering the mean of control values (tissues study) or zero E2 values (cells  
191 study) of each gel as 100. These values were further corrected based on the amount of total  
192 protein subjected to electrophoresis and the amount of total protein (per g of processed

tissue) recovered in each fraction, obtaining the final value of GLUT4 protein per g of tissue (AU/g of tissue).

#### *Data analysis*

All values are reported as means  $\pm$  SEM. The contents of GLUT4 protein, GLUT4 mRNA and GLUT4 mRNA size were quantified in at least four different experiments, using different rats from all animal groups. For comparison of the mean values, one-way ANOVA, with Student-Newman-Keuls (SNK) post-test, using GraphPad InStat version 3.01 (GraphPad Software, San Diego, CA, USA).

## **Results**

### *Animal characteristics*

The animal characteristics are presented in Table 1. Initial body weight was ~206 g, and no significant differences were found in the final body weight among the groups. Comparing controls and oophorectomized rats treated or untreated with oil, oophorectomy clearly decreased E2 levels ( $P<0.05$  vs control, ANOVA, analyzing 3 groups). Comparing all groups, E2-treated rats showed 10-fold higher estradiol levels ( $P<0.01$ ), a value similar to that observed in pregnant rats (Gonzalez *et al.*, 2003). Glycemia levels were unchanged; however, insulinemia was ~4-fold lower ( $P<0.01$ ) in Ox and Ox-O rats. The reduced insulinemia of oophorectomized rats was responsible for the increased glucose/insulin index, evincing increased insulin sensitivity. The E2-treatment decreased the glucose/insulin index to a value similar to that observed in intact control rats.

### *Effects of oophorectomy and 17 $\beta$ -Estradiol treatment in GLUT4 expression*

GLUT4 mRNA and protein in skeletal muscle and adipose tissue of control rats in proestrus (C) and oophorectomized rats treated with oil (Ox-O) or with E2 (Ox-E) for 7 days are presented in Figure 1. In muscle (Figure 1A and C), oophorectomy induced a 20% increasing ( $P<0.05$  vs C) in both GLUT4 mRNA and protein, whereas the E2 treatment induced a 33% decrease ( $P<0.01$  vs Ox-O) in these values. Differently, in adipose tissue (Figure 1B and D), oophorectomy caused a marked reduction in GLUT4 mRNA expression ( $P<0.01$ ), and the E2 treatment induced a further reduction in the mRNA ( $P<0.05$  vs Ox-O); however, these decreases in mRNA were not accompanied by changes in the GLUT4 protein content. Experiments comparing untreated 21-day oophorectomized rats with 7-day oil-treated oophorectomized rats revealed no differences between these groups.

#### *Effects of oophorectomy and 17 $\beta$ -Estradiol treatment in the subcellular distribution of GLUT4 protein*

Figure 2 shows the total protein recovery, expressed as  $\mu\text{g}$  of protein by g of tissue, and the GLUT4 protein content, expressed as AU by g of tissue, in plasma membrane (PM) and microsomal (M) subcellular membrane fractions of skeletal muscle and white adipose. Total protein recovery was unchanged among the groups in both tissues (Figure 2A and B). However, in both PM and M fractions of muscle (Figure 2C), oophorectomy increased the GLUT4 content ( $P<0.05$  vs C) and the E2 treatment reduced the GLUT4 content ( $P<0.05$  vs Ox-O) to control levels. Additionally, the PM GLUT4 content related to the total GLUT4 content, as an index of the GLUT4 translocation at the moment of the tissue harvesting, was similar among the groups. On the other hand, in adipose tissue (Figure 2D),

the GLUT4 content was unchanged among the groups in both fractions, similarly to that observed in the total GLUT4 content measured in the fat-free extract (Figure 1D).

#### *GLUT4 expression in 24-hour 17 $\beta$ -Estradiol treated L6 myotubes*

Attempting to demonstrate a direct effect of estrogen on L6 myotubes, the expression of estrogen receptors ER $\alpha$  and ER $\beta$  in these cells was first investigated (Figure 3), since there was no report concerning the isoforms expression in these cells. Immunofluorescent analysis revealed high immunoreactivity for both ER $\beta$  (Figure 3A) and ER $\alpha$  (Figure 3B) in nucleus of the myotubes (Figure 3C). Furthermore, the merged image revealed that ER $\beta$  and ER $\alpha$  coexpress in the same nucleus (Figure 3D). Additionally, weak immunoreactivity for ER $\beta$  and ER $\alpha$  can be seen dispersed in the cytoplasm, reflecting the extra-nuclear presence of the ERs.

Results of L6 myotubes cultured in different E2 concentrations for 24 hours are shown in Figure 4. The dose-response curve with E2 (0.1 to 100 nM) showed progressive increase of GLUT4 mRNA content in L6 myotubes (Figure 4A); the GLUT4 mRNA content being 5-fold higher at 100 nM when compared with 0.1 nM ( $P<0.01$ ). Furthermore, comparing with 24-hour cultured cells in the absence of E2 (E2-0), all concentrations of E2 revealed a strong reduction ( $P<0.001$ ) in GLUT4 mRNA, with the highest E2-induced GLUT4 mRNA content being a half of the content observed in the absence of E2. On the other hand, the GLUT4 protein content modulation observed in the dose-response curve showed a completely distinct pattern (Figure 4B). From zero to 100 nM E2, the L6 myotubes showed a progressive tendency to reduce the GLUT4 protein content, the value at 100 nM being significantly lower than the values observed at zero and 0.1 nM ( $P<0.05$ ).

## *GLUT4 expression in 6-day 17 $\beta$ -Estradiol treated L6 myotubes*

Attempting to investigate the effect of E2 upon the L6 cells in conditions similar to those imposed on the in vivo treatment, the cells were treated with E2 from 1 to 100 nM for 6 days, concentrations that represent values from infra- to supra-physiological concentrations of the hormone (Nogueira and Brentani, 1996). Viability of the L6 myotubes cultured for 6 days was confirmed; and the GLUT4 mRNA and protein content remained unchanged in cells cultured in the absence of E2 for 1, 2, 3 or 6 days (data not shown).

In L6 myotubes treated for 6 days (Figure 5), 100 nM E2 clearly revealed a ~25% reduction ( $P<0.05$ ) in both GLUT4 mRNA and protein contents, comparing with cells treated with zero, 0.1 and 1 nM E2. Besides, the 10 nM E2 also revealed reduced protein content as compared to zero, 0.1 and 1 nM ( $P<0.05$ ). However, with 10 nM E2, the GLUT4 mRNA was not significantly reduced (ANOVA), although it was marginally reduced when compared with zero ( $P=0.840$ , Student  $t$  Test), and significantly reduced when compared with 1 nM E2 ( $P=0.0349$ , Student  $t$  Test).

## **Discussion**

The present study shows that oophorectomy increases and 17 $\beta$ -estradiol (E2) decreases insulin sensitivity, revealing that E2 plays a role in the in vivo whole body glycemic homeostasis. These modulations were accompanied by parallel changes in the GLUT4 expression in skeletal muscle, with unchanged protein content in adipose tissue, suggesting that skeletal muscle plays a key role in the E2-induced modulation of glycemic

homeostasis. Furthermore, similar effect of E2 was confirmed in skeletal muscle L6 myotubes, definitively revealing an effect of the estrogens upon the GLUT4 gene expression, which may be involved in the insulin resistance of hyperestrogenic states, such as pregnancy and/or gestational diabetes.

Oophorectomized (Ox) rats showed increased glucose/insulin index with decreased plasma insulin concentration, pointing out an improvement in the insulin sensitivity, which may be explained by the increased GLUT4 expression observed in muscle. Primary suppression of insulin secretion might be considered in oophorectomized rats, since it has been reported that progesterone may stimulate insulin secretion (Nieuwenhuizen *et al.*, 1999, Zhu *et al.*, 1998), and that patients with Turner syndrome have reduced glucose stimulated insulin secretion (Bakalov *et al.*, 2004). If oophorectomy induces primary reduction in the insulin secretion, its effect upon insulin sensitivity must be more important, since primary defect of beta-cell secretion would increase glycemic levels. Conversely, insulin resistance has been described in postmenopausal women (Saucedo *et al.*, 2007, Godsland, 2005), but that has been related to the presence of obesity (Karelis *et al.*, 2006) or to high levels of plasma free androgens (Golden *et al.*, 2004). Thus, we can propose that oophorectomy improves peripheral insulin sensitivity, reducing plasma insulin levels as a compensatory mechanism.

E2 treatment for 7 days, achieving hormone levels similar to those observed in pregnancy, decreased insulin sensitivity and increased plasma insulin levels to values similar to those observed in intact rats in the proestrus phase. Since the plasma estradiol concentration was similar to that observed in pregnancy (Gonzalez *et al.*, 2000, Gonzalez *et al.*, 2003), we expected to detect insulin resistance, but that was not observed when compared with control rats. However, insulin resistance was clearly observed 11 days after



E2 treatment of oophorectomized rats (Gonzalez et al., 2000, Gonzalez et al., 2003). Thus, we can suppose that, although 7 days of hyperestrogenism reduced the insulin sensitivity, reversing the hypersensitivity of oophorectomized rats, this period was not enough to cause insulin resistance, as compared to intact rats. It is important to bear in mind that, in pregnancy, ovarian and placental hormones as well as the fetus are participating in the mother glycemic homeostasis. Thus, the importance of the present protocol is to exclude the participation of other insulin-sensitivity modulators, providing conditions to show that hyperestrogenism can modulate GLUT4 expression and insulin sensitivity by itself.

In muscle, oophorectomy and E2 treatment clearly induced a stoichiometric increase and decrease in GLUT4 mRNA and protein, respectively. This is perfectly in accordance with the insulin-sensitivity state of each condition. Furthermore, the plasma membrane GLUT4 protein content showed exactly the same regulation, confirming that the muscle glucose clearance capacity increases in Ox and decreases in E2-treated rats. Skeletal muscle protein synthesis rates have already been described as being increased in oophorectomized rats (Toth *et al.*, 2001); however, this result is related to all cellular proteins. In the present study, the total protein recovery in the plasma membrane and microsomal fractions (expressed by g of tissue) was unchanged among the groups, pointing out the specific modulation of the GLUT4 protein observed (also expressed by g of tissue).

On the other hand, in adipose tissue, GLUT4 mRNA decreased in both Ox and Ox-E2-treated rats with no changes in the protein content. These data point out the tissue-specific effect of estrogen upon GLUT4 gene expression, as well as the occurrence of post-transcriptional modulation. In this regard, discrepancies between GLUT4 mRNA and protein regulation have been extensively described (Klip *et al.*, 1994, Richardson *et al.*, 1991), and we have recently reported that GLUT4 mRNA poly(A) tail length changes are

involved in the post-transcriptional regulation of GLUT4 expression (Seraphim *et al.*, 2007). Besides, estrogen is the steroid hormone that is best known for its effects on mRNA stabilities of target genes (Ing, 2005). Further studies must be conducted to explain the adipose tissue modulation of GLUT4 expression, bearing in mind that the peripheral and the adipose tissue estrogen concentrations may be not related, since it is the main territory involved in the conversion of adrenal precursors.

Seeking to determine a direct effect of E2 upon GLUT4 expression in skeletal muscle, we investigated the E2 effect on L6 myotubes, where the coexpression of ER $\alpha$  and ER $\beta$  was confirmed, in accordance with what we have recently reported in gastrocnemius of mice (Barros *et al.*, 2006b). The 24-hour E2 treatment of L6 myotubes showed surprising effects upon GLUT4 expression. Comparing with cells treated without hormone, the lowest concentration ( $10^{-10}$  M) of E2 induced a strong reduction of the GLUT4 mRNA; however, progressively higher E2 concentrations showed a positive effect upon the mRNA content, revealing a biphasic effect. However, the 24-hour E2 effect upon the GLUT4 protein content was completely distinct, pointing out the occurrence of post-transcriptional modulation of GLUT4 gene expression, such as the observed in adipose tissue. After 6 days of L6 myotubes treatment, GLUT4 mRNA and protein were unchanged in low E2 concentration (zero or 0.1 nM), and clearly reduced with high E2 concentration (10 and 100 nM), differently from that observed in 24-hour treated cells, but similar to that observed in muscle from 7-day treated oophorectomized rats.

The present results in L6 myotubes definitively point out the estrogen-induced regulation of GLUT4 gene expression in skeletal muscle; but they also reveal how complex this modulation is, being time- and dose-dependent, and also involving post-transcriptional modulation. Since selective ER knockout mice revealed that ER $\alpha$  enhances, whereas ER $\beta$

suppresses the GLUT4 protein expression in muscle (Barros *et al.*, 2006b), it is reasonable to propose that the different modulations of GLUT4 observed in L6 cells may be related to a time- and dose-dependent regulation of ER $\alpha$  and ER $\beta$  expression and/or activity. Time-dependent effect of E2 upon ER expression was reported (Jeng *et al.*, 1998), but without distinction between the isoforms. On the other hand, dose-dependent regulation of E2 upon expression of ERs has revealed that a high concentration of E2 reduces ER $\alpha$  mRNA and protein content (Varayoud *et al.*, 2005). Besides, the ERs activity upon target genes has been shown that transcriptional stimulus through ER $\alpha$  starts at low E2 concentration, whereas through ER $\beta$  it occurs at high E2 concentration, with ER $\beta$  showing a transdominant effect upon ER- $\alpha$  activity (Hall and McDonnell, 1999). This way, it is plausible to suppose that at low E2 concentrations an enhancing ER $\alpha$ -dependent effect upon GLUT4 expression is dominant; whereas at high E2 concentrations a repressing ER $\beta$ -dependent effect upon GLUT4 expression is dominant.

One important point is how the ERs might be determining the GLUT4 gene regulations observed. It was demonstrated that ER can interact with other transcriptional factors such as the nuclear factor-kappa B (NF- $\kappa$ B) (Qin *et al.*, 1999, Galien and Garcia, 1997, Paech *et al.*, 1997), an inhibitor of GLUT4 expression (Ruan *et al.*, 2002, Silva *et al.*, 2005). E2 activation of ER $\alpha$ , by reducing the NF- $\kappa$ B-DNA (Nilsson *et al.*, 2001), might be increasing GLUT4 transcription, as we have already proposed for GLUT4 gene (Barros *et al.*, 2006a). On the other hand, we can not discard the possibility of the ERs to be acting as transcriptional factors. Although the classic palindromic ER-domain site is not found in the GLUT4 gene promoter, compared to other responsive elements described for ERs (Hall *et al.*, 2002), some homologues sequences are found in the GLUT4 promoter. Finally, recent

reports have described non-genomic effects of E2, involving upregulation of the mammalian target of rapamycin (mTOR) pathway (Santen *et al.*, 2005), which is known to participate in the translation process (Proud, 2007), and that could be involved in the post-transcriptional modulations observed. In order to clarify the involved mechanisms of ERs upon GLUT4 gene expression, further studies must be conducted.

In summary, the present study shows for the first time that E2 is a regulator of GLUT4 gene expression in skeletal muscle, the most important tissue involved in the insulin-stimulated glucose uptake. Long-term exposition to high concentration of E2, inhibits GLUT4 mRNA and protein expression both in vivo and in vitro. In vivo, this modulation was accompanied by decreased whole body insulin sensitivity, suggesting that hyperestrogenism may be involved in the pregnancy-induced insulin resistance and/or gestational diabetes.

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## Disclosure

We declare that there is no conflict of interest that would prejudice the impartiality of this work.

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Table 1. Characteristics of sham-operated (C), oophorectomized untreated (Ox) and oophorectomized 7-day treated with oil (Ox-O) or estradiol (Ox-E) female rats.

	C	Ox	Ox-O	Ox-E
Body weight (g)	221.7 ± 3.5	225.9 ± 8.9	230.9 ± 4.4	221.2 ± 6.6
Plasma estradiol (pg/mL)	21.2 ± 5.7	4.5 ± 1.5	5.6 ± 2.5	207 ± 25##
Plasma glucose (mM)	8.6 ± 0.53	5.5 ± 0.98	7.4 ± 0.53	7.1 ± 0.45
Plasma insulin (pg/mL)	232 ± 41	46 ± 7.1**	87 ± 19**	199 ± 36
Glucose/Insulin index	28.4 ± 7.4	87.7 ± 6.8*	62.7 ± 9.9**	27.7 ± 12.4

Glucose/Insulin index was calculated from plasma glucose (mg/dL) and insulin ( $\mu$ U/mL) concentrations. Data are mean  $\pm$  SEM of 10 (body weight) or 6 to 8 animals (metabolic-hormonal data). \* $P$ <0.05, \*\* $P$ <0.01 vs C and Ox-E; ## $P$ <0.01 vs C, Ox and Ox-O; ANOVA, Student Newman-Keuls post-test.

### Figure Legends

Figure 1. Expression of GLUT4 mRNA (*A* and *B*) and protein (*C* and *D*) in the skeletal muscle gastrocnemius (*A* and *C*) and periuterine white adipose tissue (*B* and *D*) of control (C, open bars) and 21-day oophorectomized rats treated with oil (Ox-O, closed bars) or with estradiol (Ox-E, shaded bars). Each panel shows, at the top, representative autoradiograms; at the bottom, the GLUT4/ $\beta$ -actin mRNAs values normalized to the control value (*A* and *B*), or the total GLUT4 protein content, expressed by g of tissue processed, normalized to control value (*C* and *D*). Each square of autoradiogram shows images from the same gel, and the vertical line indicates that the lanes were not contiguous. Data are expressed as arbitrary units (AU). Bars represent mean values ( $\pm$  SEM) of 5

animals per group. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs control; # $P < 0.05$  and ## $P < 0.01$  vs Ox treated with oil, ANOVA, SNK post-test.

Figure 2. Total protein content (*A* and *B*) and plasma membrane (PM) and microsomal (M) GLUT4 protein content (*C* and *D*) in the skeletal muscle gastrocnemius (*A* and *C*) and periuterine white adipose tissue (*B* and *D*) of control (C, open bars) and 21-day oophorectomized rats treated with oil (Ox-O, closed bars) or with estradiol (Ox-E, shaded bars). Total protein was expressed by g of tissue processed. PM and M GLUT4 (arbitrary units) were also expressed by g of tissue, taking into account the total protein recovery by g of tissue processed in each subcellular fraction, and after normalization of the crude results of the Western to the control values. In panels *C* and *D*, at the top, each square shows images from the same gel, and the vertical line indicates that the lanes were not contiguous. Bars represent mean values ( $\pm$  SEM) of 5 animals per group. \* $P < 0.05$  vs control and Ox treated with E2, ANOVA, SNK post-test.

Figure 3. Expression and colocalization of ER $\alpha$  and ER $\beta$  in L6-myotubes by immunofluorescence. Cells were stained for ER $\beta$  (*A*) with Cy3 (red), ER $\alpha$  (*B*) with FITC (green) and nucleus (*C*) with DAPI (blue). For colocalization (*D*), images from the same section were overlaid, and the yellow/orange color in the merged image revealed that ER $\beta$  and ER $\alpha$  coexpress in the same nucleus. The narrow shows the same nucleus in all sections. Bar = 10 $\mu$ m.

Figure 4. Expression of GLUT4 mRNA (*A*) and protein (*B*) in L6 myotubes treated in the absence (0, closed bars) or presence (open bars) of increasing concentrations (ranging from 0.1 to 100 nM) of 17 $\beta$ -estradiol (E2) for 24 hours. Representative autoradiograms are shown at the top of each panel. GLUT4/ $\beta$ -actin mRNAs values (*A*) and total cellular GLUT4 protein values (*B*) were normalized to E2-0 value. Data are expressed as arbitrary units (AU). Bars represent mean values ( $\pm$  SEM) of 4 experiments. \*\*\* $P$ <0.001 vs E2-0; ### $P$ <0.01 vs E2-0.1 nM; § $P$ <0.05 vs E2-0 and E2-0.1 nM, ANOVA, SNK post-test..

Figure 5. Expression of GLUT4 mRNA (*A*) and protein (*B*) in L6 myotubes treated in the absence (0, closed bars) or presence (open bars) of increasing concentrations (ranging from 0.1 to 100 nM) of 17 $\beta$ -estradiol (E2) for 6 days. Each panel shows, at the top, representative autoradiograms; at the bottom, the GLUT4/ $\beta$ -actin mRNAs values (*A*), or the total GLUT4 protein content values (*B*), normalized to the E2-0 value. Each square of autoradiogram shows images from the same gel, and the vertical line indicates that the lanes were not contiguous. Data are expressed as arbitrary units (AU). Bars represent mean values ( $\pm$  SEM) of 4 experiments. \* $P$ <0.05 vs E2-0, E2-0.1 and E2-1 nM, ANOVA, SNK post-test.

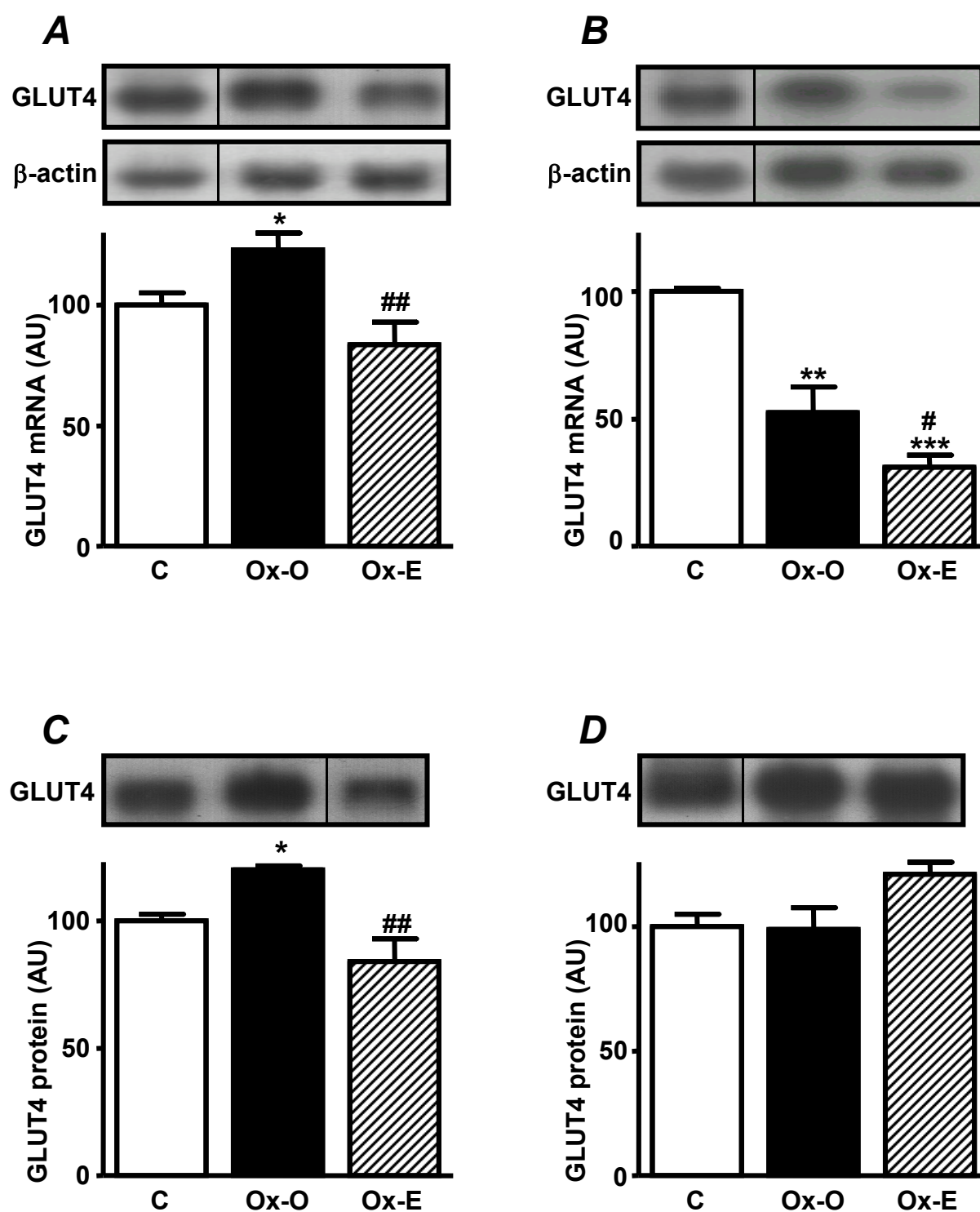


Figure 1

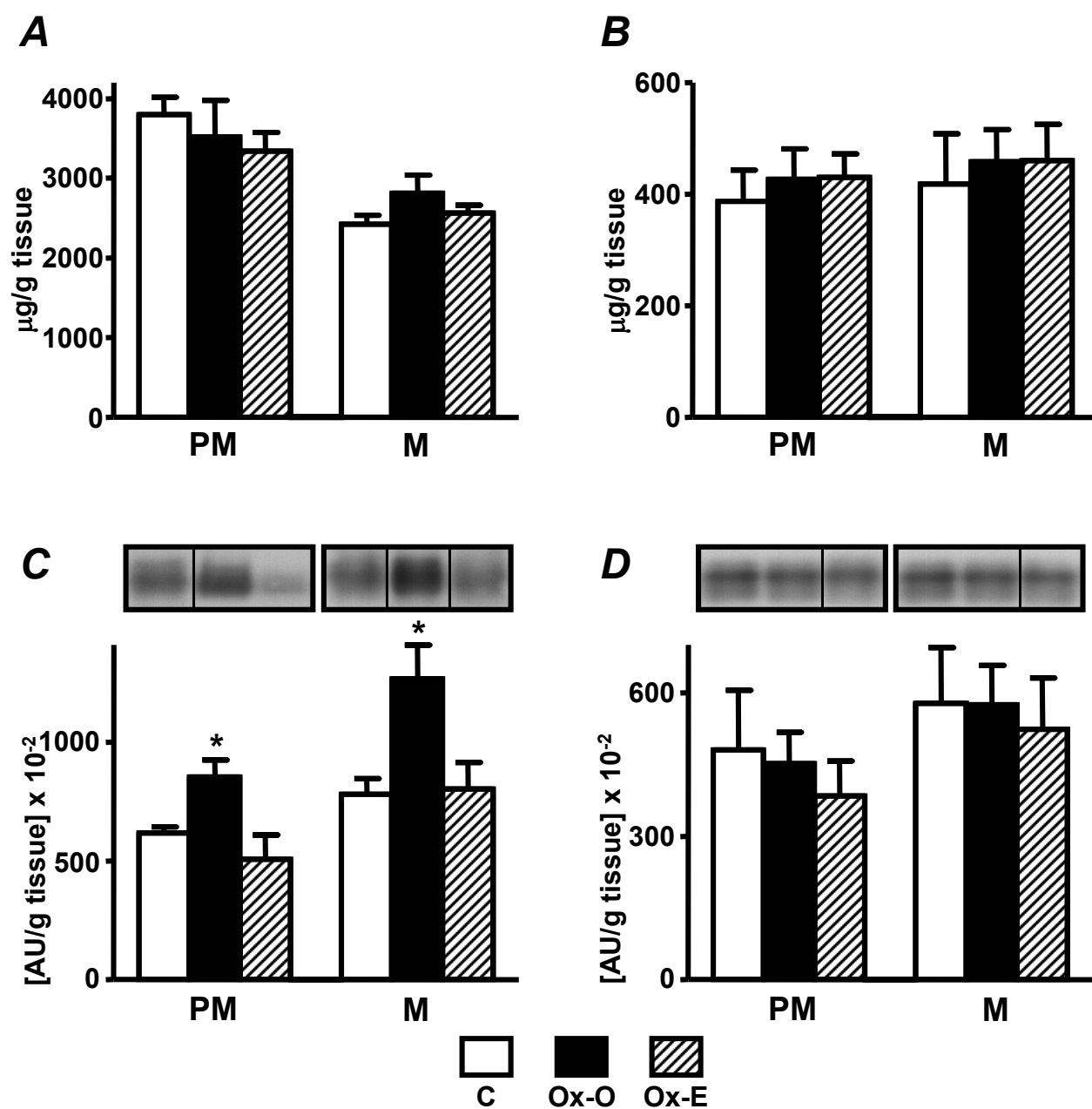


Figure 2

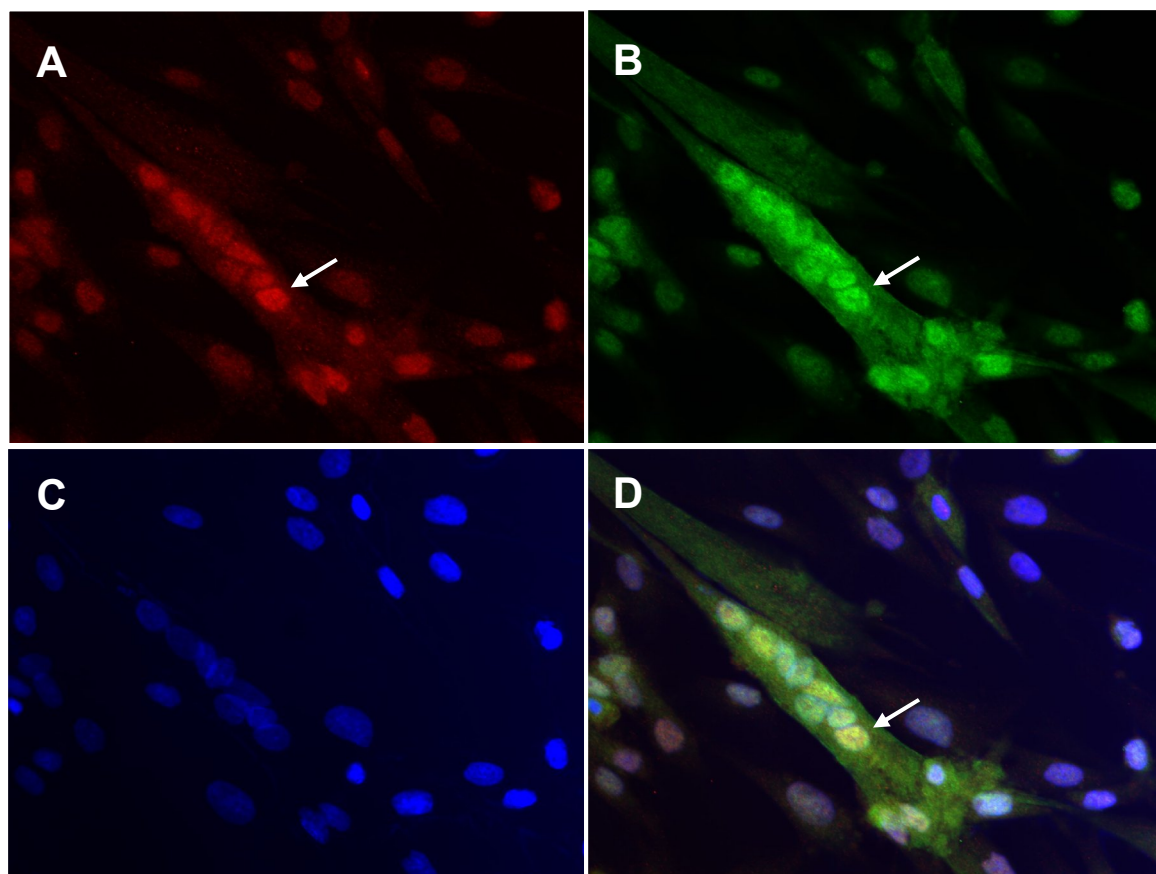


Figure 3

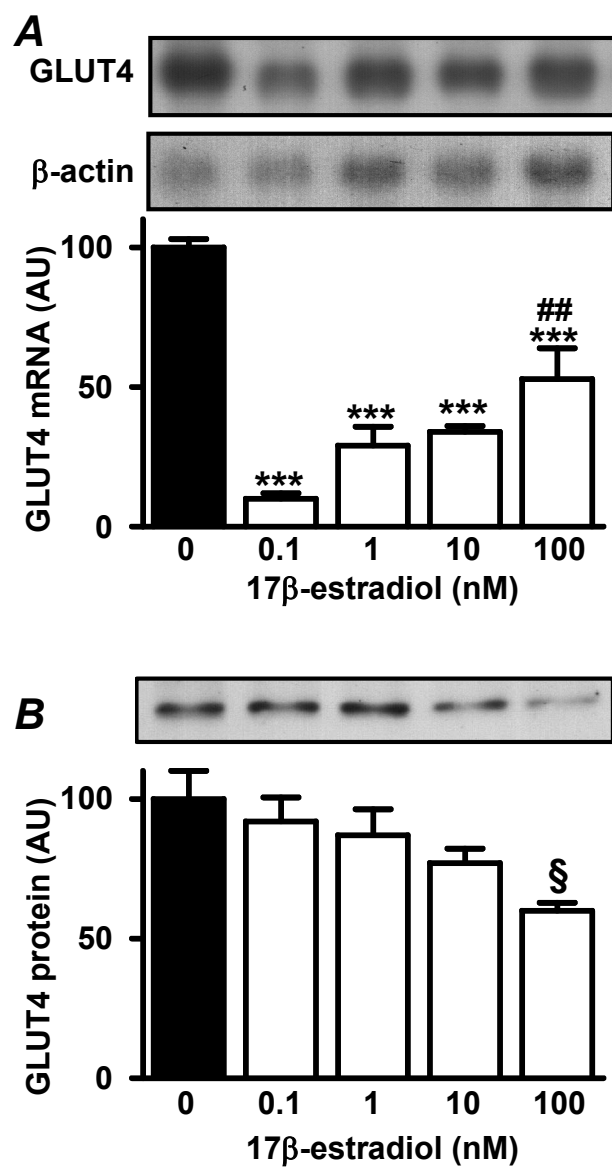
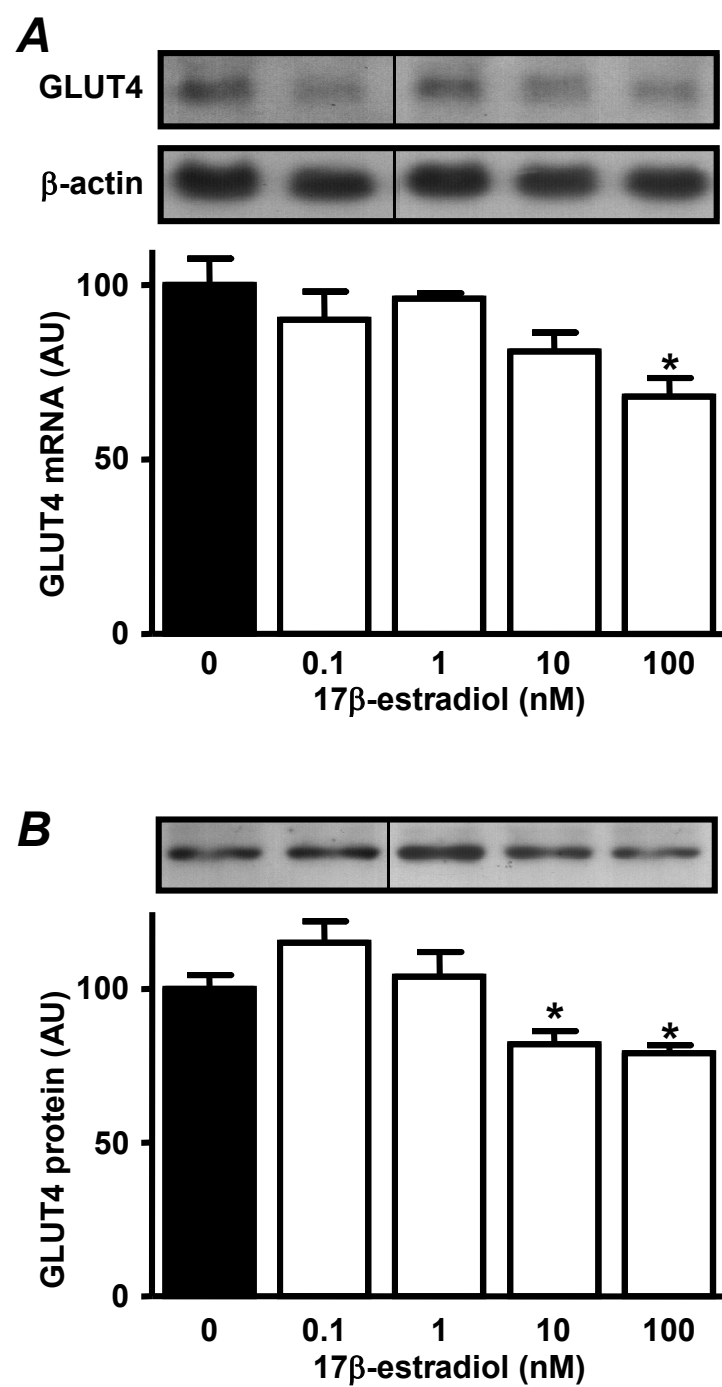


Figure 4



**Figure 5**