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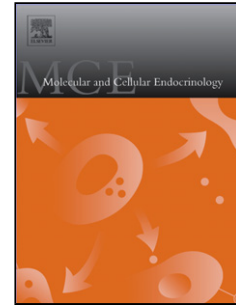
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1 **Insulin resistance of pregnancy involves**
2 **estrogen-induced repression of muscle GLUT4**

3
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14 **Keywords:** 17 Beta-estradiol, ERalpha, ERbeta, GLUT4 expression, skeletal muscle, L6
15 cell, gestational diabetes, insulin resistance

16

17 **Abstract**

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

30 **Introduction**

31 During progression of normal pregnancies, insulin sensitivity decreases, and insulin
32 resistance arises (Reece *et al.*, 1994) followed or not by diabetes (Kaaaja and Greer, 2005).
33 Gestational diabetes mellitus (GDM) has been observed in 2 to 5% of the pregnancies, and
34 its prevalence has increased in the last decade (Shaata and Groop, 2007). Besides, persistent
35 or recurrent impaired glucose homeostasis after gestational diabetes is frequent (Case *et al.*,
36 2006, Haynes *et al.*, 2003).

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

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

53 Recently, we have reported that selective estrogen receptor (ER) alpha (ER α) and
54 beta (ER β) knockout mice showed increased and reduced GLUT4 protein; respectively
55 (Barros *et al.*, 2006b). This suggests that ER α - and ER β -activity could have different
56 effects on the glucose uptake, thus indicating that estradiol participates in glycemic
57 homeostasis. In fact, the correlation between increased E2 and reduced adipose tissue
58 GLUT4 content has already been described in insulin resistant conditions such as normal
59 pregnancy, GDM or Polycystic Ovarian Syndrome (Garvey *et al.*, 1993, Okuno *et al.*,
60 1995, Rosenbaum *et al.*, 1993). However, in skeletal muscle, the most important site of
61 insulin resistance (Zorzano *et al.*, 1998, Ryder *et al.*, 2001), the GLUT4 expression in
62 pregnant subjects has not been reported yet. Only one study investigated the E2 effect on
63 muscle GLUT4 of oophorectomized rats treated or not with E2, showing that physiological
64 levels of E2 decreased GLUT4 protein in red quadriceps whereas had no effect on white
65 quadriceps (Campbell and Febbraio, 2002).

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

75 For the first time, we report that oophorectomy increased GLUT4 mRNA and
76 protein in skeletal muscle, which is reversed by the E2 treatment for 7 days. We also show
77 that L6 myotubes express both α and β isoforms of ER, and the treatment of these cells for
78 6 days with high E2 levels decreased the GLUT4 mRNA and protein expression. These
79 results together showed that a high concentration of estrogen, such as that observed in
80 pregnancy, suppresses the GLUT4 gene expression, which may participate in the
81 gestational-induced insulin resistance.

82

83 **Materials and Methods**

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

88 *Animals:*

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

99 On the day of the experiment, animals were anesthetized with sodium pentobarbital
100 (35 mg/Kg BW, *i.p.*). Blood was collected from the inferior *vena cava* for further analysis

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

109 *Cell culture:*

110 L6 myoblasts obtained from ATCC (CRL 1458) were propagated in flasks
111 (Corning, 75cm^2 TC-treated) containing Dulbecco's Modified Eagle's Medium (DMEM -
112 Gibco, Invitrogen) and 10% fetal bovine serum (FBS - Gibco, Invitrogen) in a Forma
113 Scientifica incubator at 37°C and 5% CO_2 . When cells reached 90% confluence, the cells
114 were transferred to either cell culture dishes (Corning, 100mm TC-treated), or to a chamber
115 slide system (Nunc, Lab-Tek II CC2 Chamber Slide System). The cells were allowed to
116 differentiate into myotubes by changing the medium to DMEM, 2% FBS for 6 days
117 (Yonemitsu *et al.*, 2001), and then used for the evaluation of GLUT4 expression or ERs
118 expression and colocalization.

119 After differentiation, the medium was changed to DMEM Phenol-Red Free (Gibco,
120 Invitrogen) without FBS for 24 hours, to prevent any estrogenic action (Berthois *et al.*,
121 1986). The cells were then treated in the same medium containing 2% dextran-treated FBS
122 (Sigma, C-6241) (Nogueira and Brentani, 1996) for 1, 2, 3 and 6 days, under different
123 concentrations (0.1 to 100 nM) of water soluble estradiol (Sigma, E-4389).

124 *Detection and colocalization of ER α and ER β in L6 myotubes by immunofluorescence:*

125 After the differentiation period, slides containing the myotubes were washed in PBS
126 and dried at room temperature for 20 minutes, followed by fixation in 4% PFA for 20
127 minutes, at 4°C. Following PBS washing, cells were incubated in PBS/ 1% Triton X-100
128 for 20 minutes at room temperature. To block the nonspecific binding, sections were
129 incubated in 3% BSA for 1 h at 4°C, followed by PBS washing. Sections were then
130 incubated with both anti-ER α (Santa Cruz Biotechnology, MC-20) and anti-ER β [produced
131 and donate by Prof. Jan-Åke Gustafsson, Karolinska Institute, Department of Biosciences
132 and Nutrition, Stockholm, Sweden (Saji *et al.*, 2000)] at a dilution of 1:200, in 1% BSA and
133 0.1% P40 in PBS overnight at 4°C. BSA replaced primary antibodies in negative controls.
134 After PBS washing, sections were incubated with the corresponding secondary antibodies
135 (FITC anti-rabbit and Cy3 anti-chicken, Jackson Immunoresearch) at a dilution of 1:200 in
136 PBS for 2h at room temperature. The sections were washed in PBS and lightly
137 counterstained with 4',6-diamidino-2-phenyindole (DAPI, Sigma-Aldrich) and directly
138 mounted in Vectashield antifading medium (Vector Laboratories). The sections were
139 examined under a Zeiss fluorescence microscope with filters suitable for selectively
140 detecting the fluorescence of FITC (green), Cy3 (red) and DAPI (blue). For colocalization,
141 images from the same section but showing different antigen signals were overlaid. Cells
142 where both FITC- and Cy3-conjugated secondary antibodies were present have an orange
143 or yellow color.

144 *GLUT4 mRNA analysis:*

145 Total RNA was extracted from frozen tissue (0.1g for skeletal muscle and 0.6g for
146 adipose tissue) or fresh myotubes using Trizol[®] reagent (Gibco BRL, Life Technologies,

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

156 *GLUT4 protein analysis:*

157 GLUT4 protein was assessed in a total cellular membrane fraction, as previously
158 described (Machado et al., 1993, de Carvalho Papa et al., 2002). Briefly, the white adipose
159 tissue was homogenized using a Polytron (Brinkmann Instruments, Westbury, NY) in 10
160 mmol/L Tris-HCl, 1 mmol/L EDTA, 250 mmol/L sucrose buffer, pH 7.4, and centrifuged
161 at 3,000 x g for 15 min. The fat cake was discarded, and the supernatant, a fat free extract
162 was recovered as a total cellular membrane fraction. Skeletal muscle was homogenized in
163 the same buffer, and centrifuged at 1,000 x g for 10 min. The supernatant was kept; the
164 pellet was resuspended in 1/3 of the initial volume of the buffer, and centrifuged again at
165 1,000 x g for 10 min. Both supernatants were mixed and subjected to a 150,000 x g
166 centrifugation for 75 min. The final pellet was resuspended as a total cellular membrane
167 fraction in 1 mL of buffer. L6 myotubes were homogenized in a polytron in Tris Hcl 10
168 mM, EDTA 1 mM, sucrose 250 mM buffer containing protease inhibitors (aprotinine 15
169 μ g/ml, leupeptine 5 μ g/mL e PMSF 0.07 μ 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

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

195 *Data analysis*

196 All values are reported as means \pm SEM. The contents of GLUT4 protein, GLUT4
197 mRNA and GLUT4 mRNA size were quantified in at least four different experiments,
198 using different rats from all animal groups. For comparison of the mean values, one-way
199 ANOVA, with Student-Newman-Keuls (SNK) post-test, using GraphPad InStat version
200 3.01 (GraphPad Software, San Diego, CA, USA).

201

202 **Results**

203 *Animal characteristics*

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

214

215 *Effects of oophorectomy and 17 β -Estradiol treatment in GLUT4 expression*

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

226

227 *Effects of oophorectomy and 17 β -Estradiol treatment in the subcellular distribution of*
228 *GLUT4 protein*

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

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

240

241 *GLUT4 expression in 24-hour 17 β -Estradiol treated L6 myotubes*

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

250 Results of L6 myotubes cultured in different E2 concentrations for 24 hours are
251 shown in Figure 4. The dose-response curve with E2 (0.1 to 100 nM) showed progressive
252 increase of GLUT4 mRNA content in L6 myotubes (Figure 4A); the GLUT4 mRNA
253 content being 5-fold higher at 100 nM when compared with 0.1 nM ($P<0.01$). Furthermore,
254 comparing with 24-hour cultured cells in the absence of E2 (E2-0), all concentrations of E2
255 revealed a strong reduction ($P<0.001$) in GLUT4 mRNA, with the highest E2-induced
256 GLUT4 mRNA content being a half of the content observed in the absence of E2. On the
257 other hand, the GLUT4 protein content modulation observed in the dose-response curve
258 showed a completely distinct pattern (Figure 4B). From zero to 100 nM E2, the L6
259 myotubes showed a progressive tendency to reduce the GLUT4 protein content, the value at
260 100 nM being significantly lower than the values observed at zero and 0.1 nM ($P<0.05$).

261

262 *GLUT4 expression in 6-day 17 β -Estradiol treated L6 myotubes*

263 Attempting to investigate the effect of E2 upon the L6 cells in conditions similar to
264 those imposed on the in vivo treatment, the cells were treated with E2 from 1 to 100 nM for
265 6 days, concentrations that represent values from infra- to supra-physiological
266 concentrations of the hormone (Nogueira and Brentani, 1996). Viability of the L6
267 myotubes cultured for 6 days was confirmed; and the GLUT4 mRNA and protein content
268 remained unchanged in cells cultured in the absence of E2 for 1, 2, 3 or 6 days (data not
269 shown).

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

277

278 **Discussion**

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

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

288 Oophorectomized (Ox) rats showed increased glucose/insulin index with decreased
289 plasma insulin concentration, pointing out an improvement in the insulin sensitivity, which
290 may be explained by the increased GLUT4 expression observed in muscle. Primary
291 suppression of insulin secretion might be considered in oophorectomized rats, since it has
292 been reported that progesterone may stimulate insulin secretion (Nieuwenhuizen *et al.*,
293 1999, Zhu *et al.*, 1998), and that patients with Turner syndrome have reduced glucose
294 stimulated insulin secretion (Bakalov *et al.*, 2004). If oophorectomy induces primary
295 reduction in the insulin secretion, its effect upon insulin sensitivity must be more important,
296 since primary defect of beta-cell secretion would increase glycemic levels. Conversely,
297 insulin resistance has been described in postmenopausal women (Saucedo *et al.*, 2007,
298 Godsland, 2005), but that has been related to the presence of obesity (Karelis *et al.*, 2006)
299 or to high levels of plasma free androgens (Golden *et al.*, 2004). Thus, we can propose that
300 oophorectomy improves peripheral insulin sensitivity, reducing plasma insulin levels as a
301 compensatory mechanism.

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

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

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

326 On the other hand, in adipose tissue, GLUT4 mRNA decreased in both Ox and Ox-
327 E2-treated rats with no changes in the protein content. These data point out the tissue-
328 specific effect of estrogen upon GLUT4 gene expression, as well as the occurrence of post-
329 transcriptional modulation. In this regard, discrepancies between GLUT4 mRNA and
330 protein regulation have been extensively described (Klip *et al.*, 1994, Richardson *et al.*,
331 1991), and we have recently reported that GLUT4 mRNA poly(A) tail length changes are

332 involved in the post-transcriptional regulation of GLUT4 expression (Seraphim *et al.*,
333 2007). Besides, estrogen is the steroid hormone that is best known for its effects on mRNA
334 stabilities of target genes (Ing, 2005). Further studies must be conducted to explain the
335 adipose tissue modulation of GLUT4 expression, bearing in mind that the peripheral and
336 the adipose tissue estrogen concentrations may be not related, since it is the main territory
337 involved in the conversion of adrenal precursors.

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

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

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

369 One important point is how the ERs might be determining the GLUT4 gene
370 regulations observed. It was demonstrated that ER can interact with other transcriptional
371 factors such as the nuclear factor-kappa B (NF- κ B) (Qin *et al.*, 1999, Galien and Garcia,
372 1997, Paech *et al.*, 1997), an inhibitor of GLUT4 expression (Ruan *et al.*, 2002, Silva *et al.*,
373 2005). E2 activation of ER α , by reducing the NF- κ B-DNA (Nilsson *et al.*, 2001), might be
374 increasing GLUT4 transcription, as we have already proposed for GLUT4 gene (Barros *et*
375 *al.*, 2006a). On the other hand, we can not discard the possibility of the ERs to be acting as
376 transcriptional factors. Although the classic palindromic ER-domain site is not found in the
377 GLUT4 gene promoter, compared to other responsive elements described for ERs (Hall *et*
378 *al.*, 2002), some homologues sequences are found in the GLUT4 promoter. Finally, recent

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

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

391

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399

400 **Disclosure**

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

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587 Table 1. Characteristics of sham-operated (C), oophorectomized untreated (Ox) and
588 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

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

593

594 **Figure Legends**

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

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

606

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

618

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

625

626 Figure 4. Expression of GLUT4 mRNA (*A*) and protein (*B*) in L6 myotubes treated in the
627 absence (0, closed bars) or presence (open bars) of increasing concentrations (ranging from
628 0.1 to 100 nM) of 17 β -estradiol (E2) for 24 hours. Representative autoradiograms are
629 shown at the top of each panel. GLUT4/ β -actin mRNAs values (*A*) and total cellular
630 GLUT4 protein values (*B*) were normalized to E2-0 value. Data are expressed as arbitrary
631 units (AU). Bars represent mean values (\pm SEM) of 4 experiments. *** P <0.001 vs E2-0;
632 ## P <0.01 vs E2-0.1 nM; § P <0.05 vs E2-0 and E2-0.1 nM, ANOVA, SNK post-test..

633

634 Figure 5. Expression of GLUT4 mRNA (*A*) and protein (*B*) in L6 myotubes treated in the
635 absence (0, closed bars) or presence (open bars) of increasing concentrations (ranging from
636 0.1 to 100 nM) of 17 β -estradiol (E2) for 6 days. Each panel shows, at the top,
637 representative autoradiograms; at the bottom, the GLUT4/ β -actin mRNAs values (*A*), or the
638 total GLUT4 protein content values (*B*), normalized to the E2-0 value. Each square of
639 autoradiogram shows images from the same gel, and the vertical line indicates that the
640 lanes were not contiguous. Data are expressed as arbitrary units (AU). Bars represent mean
641 values (\pm SEM) of 4 experiments. * P <0.05 vs E2-0, E2-0.1 and E2-1 nM, ANOVA, SNK
642 post-test.

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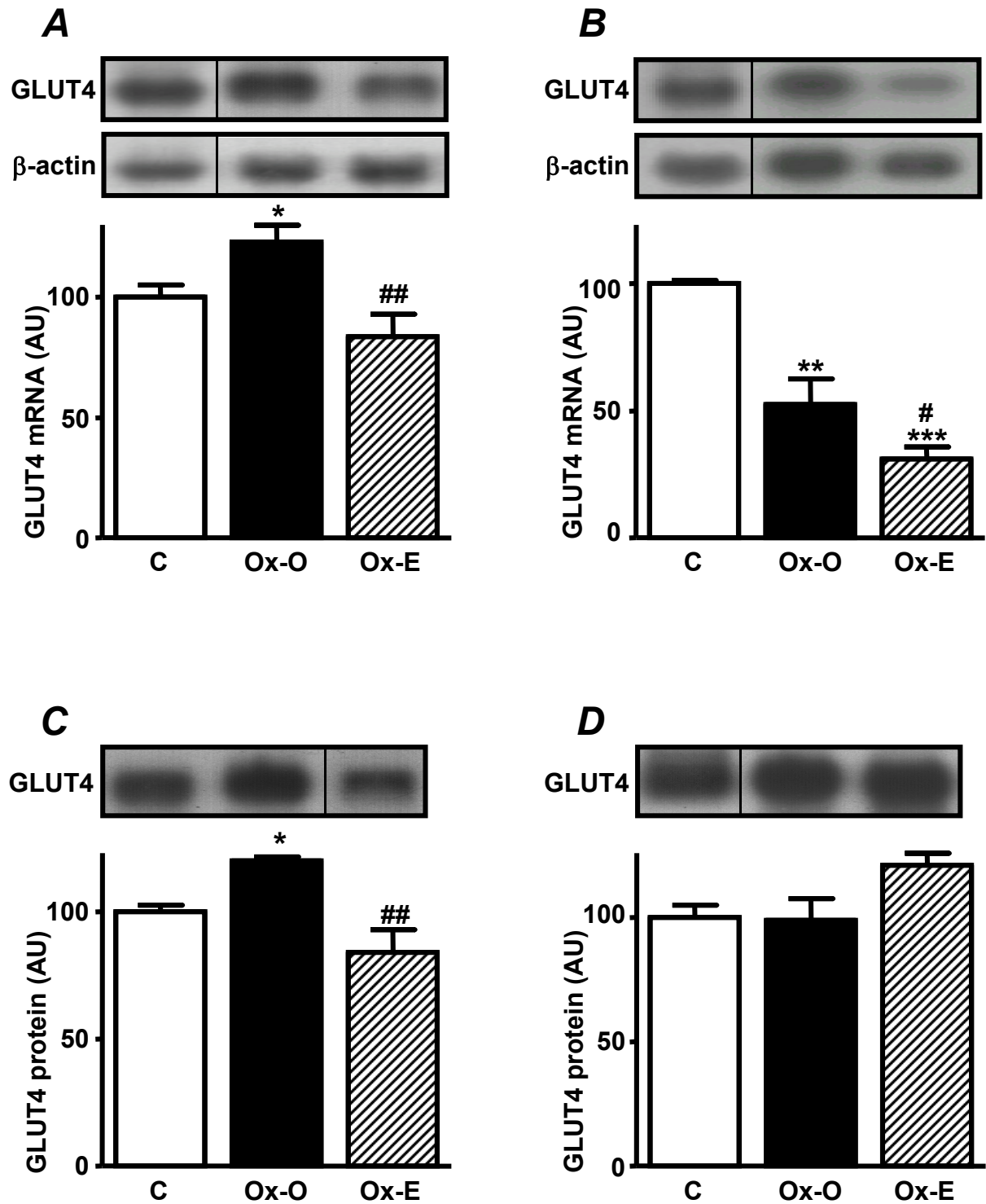


Figure 1

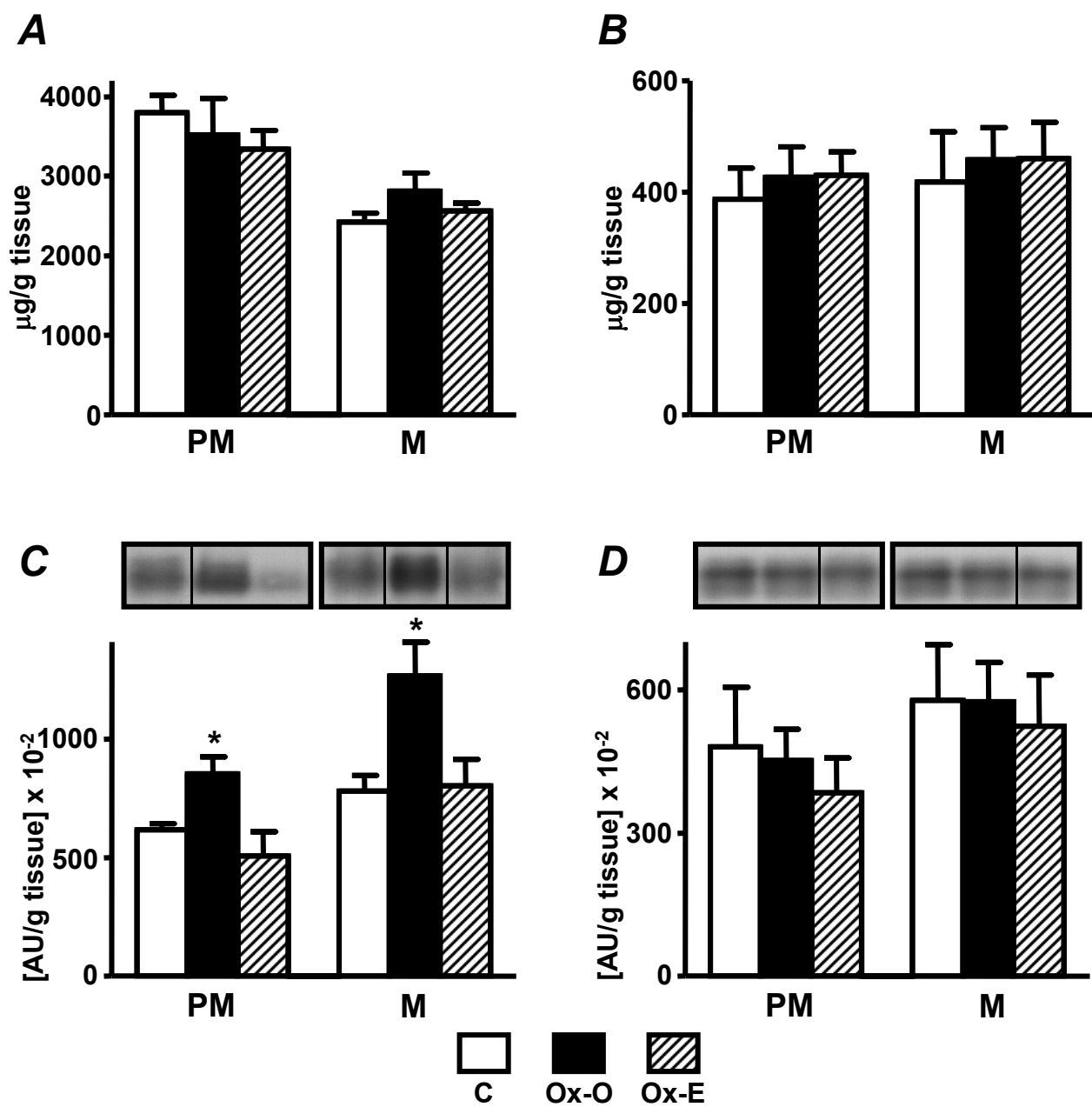


Figure 2

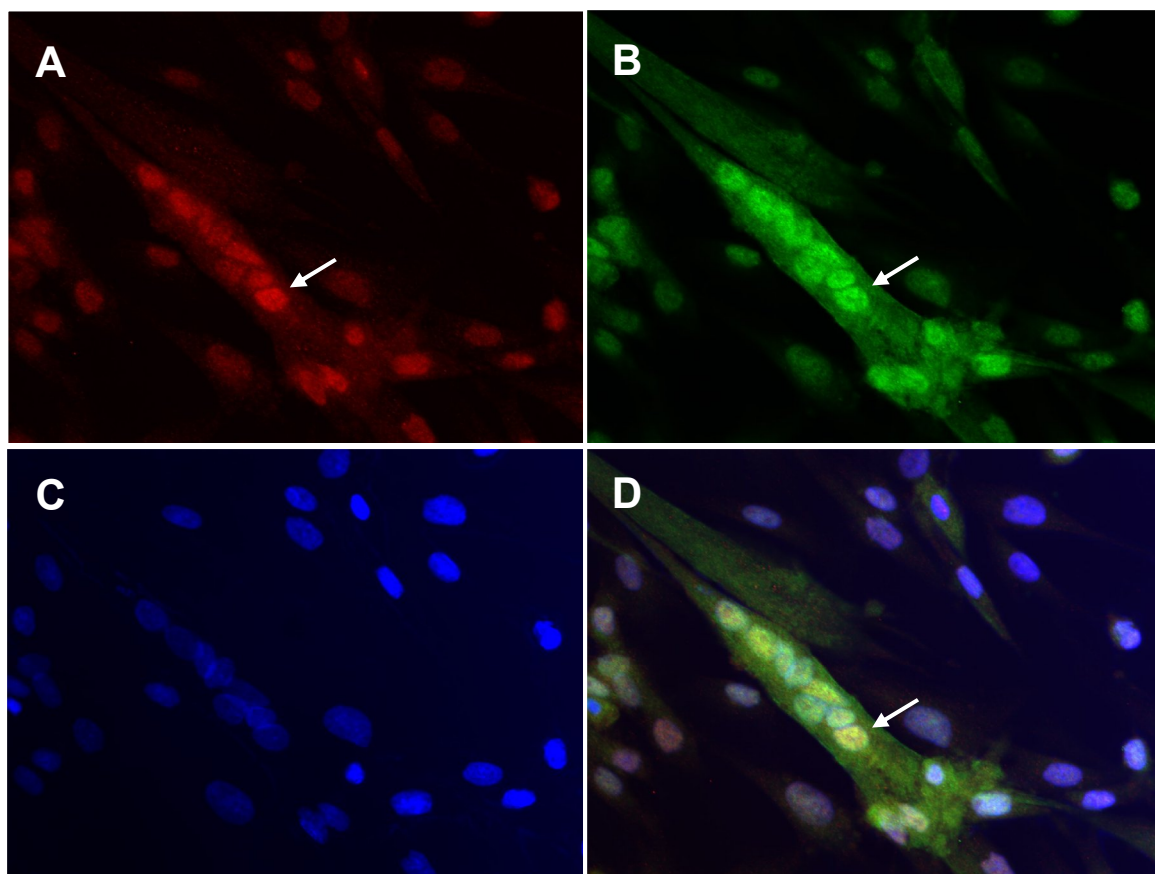


Figure 3

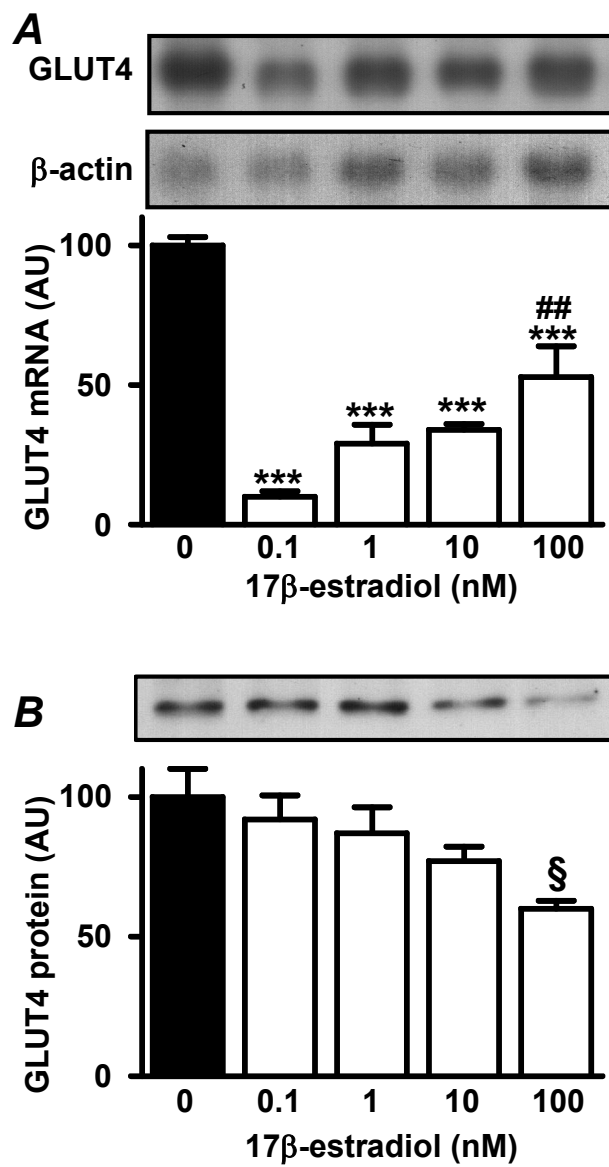


Figure 4

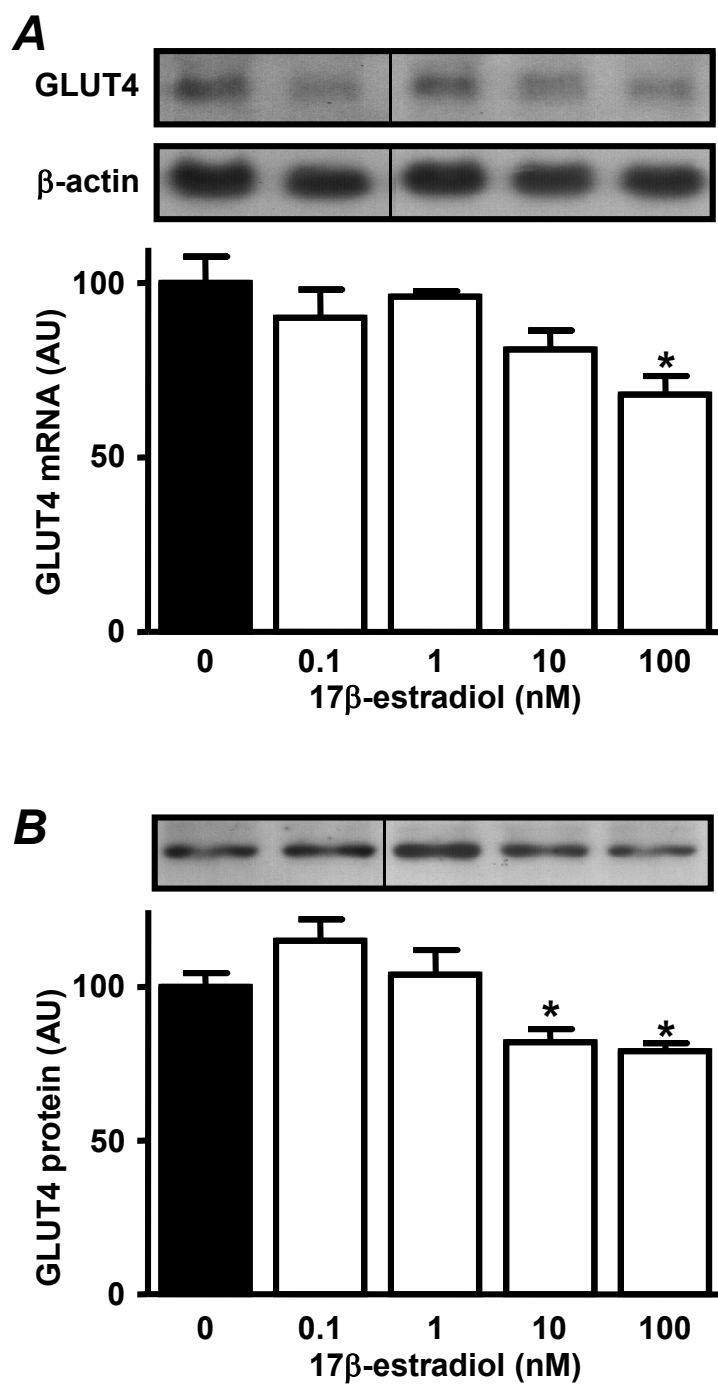


Figure 5