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17β-Estradiol treatment is unable to reproduce p85α redistribution associated with gestational insulin resistance in rats

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ABSTRACT

Maternal metabolic adaptations are essential to ensure proper fetal development. According to changes in insulin sensitivity, pregnancy can be divided into two periods: early pregnancy, characterized by an increase in maternal insulin sensitivity, and late pregnancy, in which there is a significant increase in insulin resistance. The aims of the present work were two-fold: firstly, the molecular mechanisms associated with the development of pregnancy-related insulin resistance in peripheral tissues, mainly retroperitoneal adipose tissue and skeletal muscle, were studied in pregnant rats at 6, 11, and 16 days gestation. Secondly, the role of 17β-estradiol in this process was elucidated in an animal model consisting of ovariectomized rats treated with 17β-estradiol to mimic plasma gestational levels. The results support the conclusion that retroperitoneal adipose tissue plays a pivotal role in the decrease in insulin sensitivity during pregnancy, through a mechanism that involves p85α redistribution to the insulin receptor and impairment of Glut4 translocation to the plasma membrane. Treatment with 17β-estradiol did not reproduce the molecular adaptations that occur during pregnancy, suggesting that other hormonal factors presents in gestation but absent in our experimental model are responsible for p85α redistribution to the insulin receptor.

1. Introduction

Insulin resistance is defined as a complex nutritional-metabolic state characterized by a lowered sensitivity of target tissues (liver, skeletal muscle, and adipose tissue) to the physiological effects of insulin [1]. It is considered the main metabolic feature of type 2 diabetes, with several studies indicating that insulin resistance generally precedes disease onset [2–4]. However, there are physiological situations in which insulin sensitivity is decreased without clinical implications. For example, it has been widely known for almost 50 years ago that normal pregnancy in the rat is associated with hyperinsulinemia and a progressive decline in insulin sensitivity (for review, [5]). In this respect, it was shown previously [6–12] that pregnancy can be divided into two periods: early pregnancy, characterized by an increase in insulin sensitivity in maternal tissues, and late pregnancy, in which insulin sensitivity is decreased. This critical physiological adaptation of maternal metabolism ensures an adequate supply of nutrients to the foetus. Moreover, these maternal metabolic adaptations disappear after delivery, suggesting that gestational hormones play a pivotal role in this condition [13].

Insulin signaling is initiated after the binding of insulin to its surface receptor: insulin receptor (IR) tyrosine kinase undergoes tyrosine phosphorylation of its β-subunit and kinase activation. The activated IR is essential for mediating insulin action, including the phosphorylation of substrates adaptor proteins, principally insulin receptor substrate-1 (IRS-1) and IRS-2, on tyrosine residues that interact with SH2-domain-containing proteins to generate downstream signals [14,15]. Phosphatidylinositol 3-kinase (PI3-K) bears the SH2 domain on its regulatory subunit (p85α) and is activated following interaction with IRS proteins. PI3-K, in turn, phosphorylates inositol phospholipids at the 3-hydroxy position of the inositol ring, resulting in the generation of essential products for insulin metabolic mechanisms, such as Glut4 translocation, glycogen synthase activation, and insulin-induced mitogenesis.

Several findings suggest that insulin resistance during pregnancy is exerted through an impairment of the insulin cascade at the level of the IR or IRS proteins [9–11,16–18], and that serine phosphorylation of IRS-1 reduces the protein's ability to act as an IR substrate. These phosphorylation events occur on multiple sites of the protein. In particular, the phosphorylation of serine 612, a residue located close to the C-terminal end of IRS-1, plays a signifi-

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cant role in insulin resistance at the level of glucose uptake in type 2 diabetes [19,20]. Interestingly, p85α expression in the skeletal muscle of women with insulin-resistant gestational diabetes mellitus was found to be increased during late pregnancy [17], while in transgenic mice expression of the protein appears to improve insulin sensitivity [21]. However, the role of increased p85α in the insulin resistance of gestational diabetes mellitus is unclear. It is also unknown whether insulin signaling downstream from PI3-K, leading to Glut4 translocation, is inhibited in skeletal muscle during gestational diabetes mellitus [22]. The levels of Glut4 in skeletal muscle are unchanged in women during pregnancy and in women with gestational diabetes mellitus. In different insulin-resistant states, however, reduced glucose transport in skeletal muscle is closely associated with impaired Glut4 translocation rather than a change in total Glut4 protein levels [23]. In addition, the results of studies in rats and cell culture suggest that increased IRS-1 Ser/Thr phosphorylation inhibits PI3-K activity as well as Glut4 translocation, but the factors responsible for serine kinase activity have been difficult to demonstrate under physiological conditions (for review, [24]).

Estrogens play an important role in tissue metabolism through a specific regulation of several intracellular pathways. The action of 17β-estradiol in living cells is mediated by a complex signaling network rather than a single uniform mechanism. In addition to its role as a regulator of gene transcription, mediated by the classical nuclear estrogen receptor (ER), estradiol has rapid non-genomic actions through membrane ERs (for review, [25]). Indeed, ERs located in plasma membrane have been described in reproductive and non-reproductive tissues, suggesting the importance of this steroid not only in the regulation of the reproductive cycle, but also in insulin sensitivity, skeletal muscle and adipose tissue metabolism, and neuronal activity (for review, [26–28]). The importance of these rapid non-genomic signals accounts for the tremendous interest in elucidating the structural basis and mechanisms that mediate extranuclear ER signaling.

Previously [10,11,29,30], we have shown that, in ovariectomized rats, the administration of different doses of 17β-estradiol reproduces fluctuating plasma levels of the hormone and promotes changes in insulin sensitivity analogous to those occurring throughout pregnancy. The present study expands this work with two specific aims: firstly, tissue variations of p85α and the involvement of this PI3-K subunit in the development of insulin resistance during pregnancy were studied by focusing on insulin-stimulated coupling of IRS-1 and IR, as well as total and plasma-membrane levels of Glut4 in both skeletal muscle and adipose tissue during pregnancy. Secondly, we sought to elucidate the effect of 17β-estradiol on the proteins and interactions identified in the first series of experiments.

2. Material and methods

2.1. Animals

Virgin female Wistar rats (from the Biotery of the Faculty of Medicine, University of Oviedo) weighing 250–280 g, and kept under standard conditions of temperature (23 ± 3 °C) and humidity (65 ± 1%), and a regular lighting schedule of 12 h light/dark cycle (08.00–20.00 h) were used. The animals were fed with a standard diet (Panlab A04) and all of them had free access to water. All experimental manipulations were performed between 09.30 h and 12.30 h. Animal experiments were approved by the University of Oviedo Ethic Committee following the Guiding Principles for Research Involving Animals and Human Beings Recommendations from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals.

Table 1
Schedule for 17β-estradiol hormonal treatment. From day −4 the rats were injected subcutaneously, every 12 h (09:00 h and 21:00 h) with 0.1 ml of a suspension of 17β-estradiol (according *A* = 1.3 μg/kg body wt or suspension *B* = 0.65 μg/kg body wt) in olive oil/ethanol (3:2, v/v) in order to reproduce gestational estradiol plasma levels as previously described [24–27]. The control group (V) was injected with vehicle (olive oil/ethanol 3:2, v/v) in parallel.

Days→	−7	−6	−5	−4	−3	−2	−1	0	1	2	3	4
Vehicle	–	–	–	V	V	V	V	V	V	V	V	V
Estradiol	–	–	–	A	A	A	A	A	A	A	A	A

Days→	5	6[a]	7	8	9	10	11[a]	12	13	14	15	16[a]
Vehicle	V	V	V	V	V	V	V	V	V	V	V	
Estradiol	A	B	B	B	B	B	A	A	A	A	A	

V = vehicle = olive oil/ethanol 3:2 (v/v); *A* = 1.3 μg/kg body wt; *B* = 0.65 μg/kg body wt.
[a] Day of animals were killed for study.

To obtain pregnant animals, three months old rats were placed in sets of three in separate cages with one male per cage. Pregnancy was verified by daily vaginal smears (09.30 h) for the presence of sperm. When sperm was found this was counted as day 0 of pregnancy. Rat pregnancy last three weeks. Pregnant females (G) were divided randomly into three groups (5 animals/group) and were sacrificed after 6, 11 and 16 days of gestation (G6, G11 and G16). We select these days because in previous studies [11,29] we have demonstrated that there were characteristic changes in the insulin sensitivity in rat throughout pregnancy. In this sense, we showed that gestation can be divided into two periods: early gestation which is characterized by an increase in the sensitivity to insulin action in the maternal tissues (since days 0 to 10–11 of pregnancy) and later gestation characterized by a decrease in this sensitivity (since days 10–11 to 20–21). A pregnant group treated with vehicle (olive oil/ethanol 3:2, v/v) was following in parallel in order to state that treatment with vehicle is not capable in inducing the molecular adaptations observed during pregnancy. We did not found any differences between both groups of pregnant animals.

Non-pregnant rats were ovariectomized (day −7) and were allowed 3 days to recover from the stress of surgery and to allow their hormone levels to decrease. From day −4 the rats were injected subcutaneously, every 12 h (09:00 h and 21:00 h) with 0.1 ml of a suspension of 17β-estradiol (according *A* = 1.3 μg/kg body wt; or suspension *B* = 0.65 μg/kg body wt) (Sigma Chemical Co., St. Louis, MO, USA) in olive oil/ethanol (3:2, v/v) (E group) during 10, 15 or 19 days of experimentation in order to reproduce gestational estradiol plasma levels as previously described [10,11,29,30] (Table 1). The control group (V) was injected with vehicle (olive oil/ethanol 3:2, v/v) in parallel. During the experimental treatment, the animals were fed with a standard diet (Panlab A04) and all of them had free access to water until the time of killing. Five animals of each group were killed after 6, 11 and 16 days of experiment.

2.2. Euglycemic–hyperinsulinemic clamp

After 12 h of fasting, the day of killing pregnant or treated animals were anaesthetized with sodium pentobarbital (50 mg/kg) and euglycemic–hyperinsulinemic clamp experiments were performed as previously described [10,31]. Body temperature was maintained at 37–38 °C with heating lamps. Left saphenous vein was catheterized for insulin and glucose infusion. A blood sample for the determination of basal blood glucose was collected from the tail. Plasma glucose was measured using an Accutrend System (Accutrend Alpha®, Roche Diagnostic S.L., Barcelona, Spain). Insulin (Biosynthetic Human Insulin Actrapid, Novo Nordisk, Basgsvaerd, Denmark) was dissolved in 0.9% NaCl containing 0.28% bovine serum albumin (Sigma Chemical Co., San Luis, USA) and

0.125‰ of heparin (sodic heparin 1%), and was infused at a constant rate of 20 μl/min (0.4 UI kg^{-1} h^{-1}) in left saphenous vein. The blood glucose level clamped at the level measured in the basal state by a variable infusion of glucose through the saphenous vein with a Precidor pump (Precidor Type 5003 Infusion Pump, INFORS AG, Switzerland). The infusion of exogenous glucose (12% solution) dissolved in 0.9% NaCl, was initiated 5 min after insulin infusion. Then, 25 μl of blood was sampled from the tail every 5 min and plasma glucose concentration was determined with an Accutrend System. Adjustments in the exogenous glucose infusion rate were made to maintain euglycemia by altering the percent dial of the Precidor pump depending on the changes in blood glucose concentrations observed. The duration of the experiment was about 1 h and the euglycemic clamp was easily reached 30–40 min after the beginning of insulin infusion. The total amount of blood necessary for monitoring the blood glucose concentration was quite acceptable for an animal of this size (0.5 ml).

2.3. Insulin and estradiol plasma level

In order to analyze insulin and estradiol plasma levels, blood samples were collected from the jugular vein into heparinized tubes before and after clamp experiment, centrifuged at 3000 rpm during 20 min at 4 °C and plasma was immediately drawn off and stored frozen at −20 °C until assayed. Plasma insulin was measured by RIA using a DGR Instruments GmbH (Germany) kit for rat insulin. The sensitivity of the assay was 0.1 ng/ml, and the intra-assay coefficient of variation was 9.32%. Plasma 17β-estradiol was measured by RIA using Immuchen kits of cover tubes (ICN Pharmaceuticals Inc., Costa Mesa, USA). The assay sensitivity was 10 pg/ml, and the intra-assay coefficient of variation was 12.26%. The samples were assayed in duplicate. All samples were measured on the same day.

2.4. Plasma-membrane preparation and Glut4 immunoblotting

Finally, samples of hindlimb skeletal muscle (flexor digitorum superficialis, extensor digitourm longus and extensor digitorum lateralis) and retroperitoneal adipose tissue were immediately frozen in liquid nitrogen for future experiments. Our previous experience [9,10,18] and the results from other authors [32] indicate to us that in retroperitoneal fat the response to estrogens and progesterone is different than in mesenteric or gonadal fat. On the other hand, in all our previous studies that have been made using retroperitoneal adipose tissue, we have studied the role of estradiol on intracellular insulin signaling during pregnancy and during estradiol treatment. Therefore, in this work which entails the further step towards the understanding of the role of estrogen on sensitivity to insulin action, we believe we must continue to use the same fatty deposits than in previous works.

Cell membrane preparations from a pool of the skeletal muscle and adipose tissue were performed by a modification of the method described by Hirshman et al. [33] and used by us previously [12]. Briefly, a total of 500 mg of each tissue were homogenized with a Polytron operated at maximum speed for 30 s at 4 °C in a buffer containing 100 mM Tris (pH 7.5), 2 mM EDTA (pH 8.0), and 255 mM sucrose (pH 7.6). The homogenate was then centrifuged at 1000 × *g* for 5 min, and the resulting supernatant was centrifuged again at 48,000 × *g* for 20 min. The pellet from this centrifugation was used for preparation of the membrane fraction, which was enriched in the membrane marker α1 subunit of Na^+–K^+-ATPase. The membrane fraction was prepared by resuspending the pellet in buffer 1 (20 mM HEPES, 250 mM sucrose, pH 7.4). An equal volume of a solution containing 600 mM KCl and 50 mM sodium pyrophosphate was added, and the mixture was vortexed, incubated for 30 min on ice, and then centrifuged for 1 h at 227,000 × *g* over a 36% sucrose cushion in buffer 1. The resulting interface and the entire buffer above it were collected, diluted in an equal amount of buffer 1, and centrifuged for 1 h at 227,000 × *g*. The resulting pellet was used as the membrane fraction. The protein content was performed by the Bradford dye-binding method [34]. To determine Glut4 in plasma membrane, aliquots of 30 μg were separated using polyacrylamide (10% SDS-PAGE) gels. The proteins were transferred to nitrocellulose membranes and Western blot analysis was performed using a 1:1000 dilution of polyclonal antibody against Glut4 (sc-7938, Santa Cruz Biotech., Inc.). Finally, after stripping another Western blot assay as performed against α1 subunit of Na^+–K^+-ATPase (sc-16043, Santa Cruz Biotech., Inc.) to ensure the purity of plasma-membrane fractions. This protein was also used as loading control. To avoid artefacts due to differences on protein load, Glut4 protein measurement was normalised to Na^+–K^+-ATPase analysis.

2.5. Immunoprecipitation and Western blotting

The samples of skeletal muscle and retroperitoneal adipose tissue of each animal were washed with ice-cold PBS and homogenized immediately in 3 ml of lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet® P40 Roche Diagnostic, 0.05% sodium deoxycholate and 0.1% ortovanadate 1 M) at 4 °C. The extracts were centrifuged at 12,000 × *g* at 4 °C in order to remove insoluble material [9,29,30]. Total protein content was determined with Bradford dye-binding method [34] using the Bio-Rad reagents and BSA as standard. To ensure that the proteins were in a linear range of detection, preliminary experiments were conducted to determine that the amount of homogenate protein load was within a range that resulted in a proportionate change in signal intensity as the amount of protein loaded was varied.

To determine the level of total p85α and total Glut4, similar size aliquots (20 μg total protein) were subjected to 7% SDS-PAGE, transferred to nitrocellulose membranes and Western blot analysis were performed. Briefly, membranes were incubated 1 h t in blocking buffer (Tris–NaCl–Tween20 TNT–7% bovine albumin serum) and incubated overnight using a 1:1000 dilution of anti-Glut4 antibody. Membranes were washed and incubated with an antirabbit polyclonal antibody coupled to HRP (sc-2004 Santa Cruz, diluted 1:15,000). Proteins were detected with the ECL reagent (Amersham Pharmacia Biotech.) according to the manufacturer's instructions and using autoluminography on Kodak X-Omat film. Later, the membranes were incubated during 40 min in stripping buffer (50 ml Tris–HCl 62.5 mM pH 6.8, 1 g SDS, 0.34 ml β-mercaptoethanol) at 55 °C and another Western blot analysis was performed using a 1:1500 dilution of polyclonal antibody against p85α (sc-423, Santa Cruz Biotech., Inc.). Adult rat skeletal muscle protein was used as a positive control. To verify equal protein load, all the membranes were routinely stained with Ponceau Red. Moreover, a β-actin Western blot was performed (sc-47778, Santa Cruz Biotech., Inc.) [9,11,29,30].

The aqueous fraction containing 500 μg protein from crude homogenate for muscle and adipose tissue was used for immunoprecipitation (IP) with polyclonal antibody against the insulin receptor substrate 1 (IRS-1) (sc-559, Santa Cruz Biotech., Inc.). The immune complexes were precipitated with protein G-agarose beads (Roche Diag.) overnight at 4 °C in a rocking platform and centrifuged 2 min at 13,000 × *g*. The supernatant (supernatant A) was IRS-1 depletioned and we reserved it for subsequent IP. The pellet obtained were washed several times in wash buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 0.1% Nonidet® P40, 0.05% sodium deoxycholate, 0.1% ortovanadate 1 M), and finally were centrifuged 2 min at 13,000 × *g*. After washing, the pellet was suspended in protein loading buffer (250 mM Tris–HCl pH 6.8, 8% SDS, 8 mM EDTA, 35% glycerol, 2.5% β-mercapto-ethanol, Bromophenol Blue) and heated in a boiling water bath for 5 min. Efficacy of each IP was confirmed by Western blot analysis of a second round of IP,

which yielded no detectable IRS-1 protein [9,11,29,30]. Samples were resolved by 7% SDS-PAGE gel and transferred to nitrocellulose membranes. Western blotting was performed as described above using a 1:1500 dilution of anti phospho-IRS-1 (Ser612) (2386, Cell Signalling Technology, Inc.) and proteins were detected with the ECL. Later, the membranes were incubated in stripping buffer and another Western blot analysis against p85α was performed as described above.

The supernatant A (IRS-1 depletioned), was used to perform a new IP using, in this case, an antibody against the IR β-subunit (sc-711, Santa Cruz Biotech., Inc.). After immunocomplex precipitation by centrifugation, samples were resolved by 7% SDS-PAGE gel and transferred to nitrocellulose membranes. Western blotting was performed as described above using a 1:1500 dilution polyclonal antibody against p85α.

The bands were quantified using a digital scanner (Nikon AX-110) and NIH Image 1.57 software.

2.6. Statistics

Data are expressed as means ± S.E.M. Previously, we evaluated the Gaussian distribution of each variable. Thereafter, the comparisons were made using an analysis of variance and the Student–Newman–Keuls test. A P value of 0.05 was considered significant. Statistical analysis was performed using SPSS v 15.0 for Windows.

3. Results

3.1. Plasma estradiol levels

Fig. 1 shows the plasma estradiol levels of pregnant and hormone-treated animals. As previously reported [10], plasma estradiol levels in the E group were similar to those achieved in pregnant rats at 6, 11, and 16 days of gestation.

3.2. Euglycemic–hyperinsulinemic clamp studies

During pregnancy, the complex regulation of glucose metabolism supports fetal development [9,18]. Table 2 lists the glucose and insulin values before and after clamping. Fig. 2

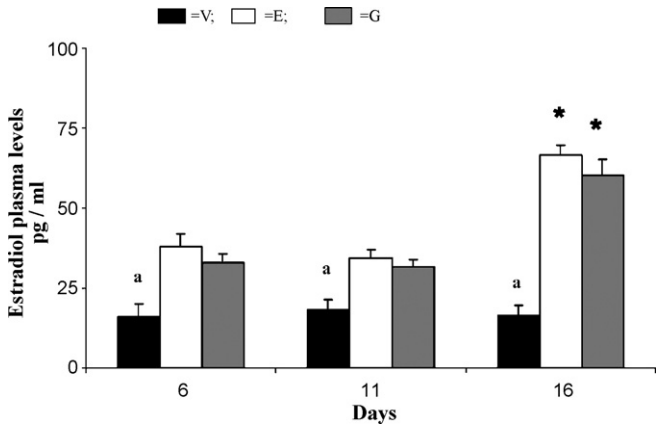

Fig. 1. Changes in 17β-estradiol plasma levels during pregnancy and 17β-estradiol treatment. Pregnant rats (G), ovariectomized rats treated with vehicle (V) and ovariectomized rats treated with estradiol (E). Mean ± S.E.M. for 5 animals. Only significant differences are shown. Intraday comparisons: a = V vs E, G; interday comparisons: ✱ = 16 vs 6, 11.

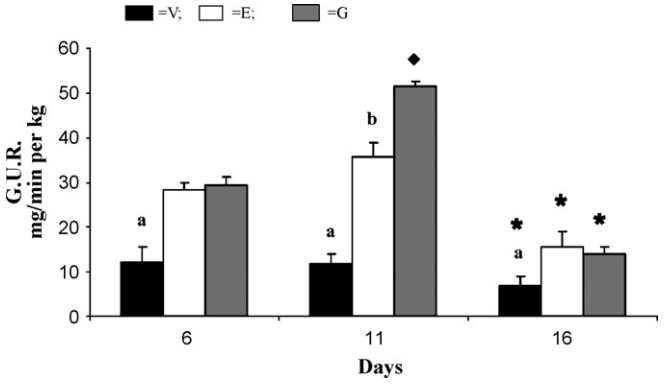

Fig. 2. Changes in glucose infusion rates (GUR) during pregnancy and 17β-estradiol treatment. Pregnant rats (G), ovariectomized rats treated with vehicle (V) and ovariectomized rats treated with estradiol (E). Glucose infusion rate was assessed as the mean values from 40 to 60 min during euglycemic–hyperinsulinemic clamp experiments. Mean ± S.E.M. for 5 animals. Only significant differences are shown. Intraday comparisons: a = V vs E, G; b = E vs G; interday comparisons: ✱ = 16 vs 6, 11; ◆ = 11 vs 6.

Table 2
Fasting blood glucose and blood glucose after clamp experiment, fasting serum insulin and serum insulin after clamp experiment of pregnant (G), vehicle (V) and estradiol treated rats (E) during experiment.

	Day 6	Day 11	Day 16	Interday comparisons
Fasting blood glucose (mg/dl)				
V	108.77 ± 4.01	110.00 ± 1.02	113.16 ± 2.38	
E	118.50 ± 5.31	108.33 ± 4.69	117.51 ± 4.44	
G	141.68 ± 6.24	145.56 ± 6.36	111.70 ± 4.12	16 vs 6, 11
Intraday comparisons	G vs V, E	G vs V, E		
Blood glucose post clamp (mg/dl)				
V	109.26 ± 3.21	110.53 ± 1.23	112.79 ± 2.69	
E	119.53 ± 4.36	110.45 ± 5.36	115.89 ± 3.48	
G	140.87 ± 5.49	144.58 ± 5.23	112.61 ± 3.73	16 vs 6, 11
Intraday comparisons	G vs V, E	G vs V, E		
Fasting serum insulin (ng/ml)				
V	0.67 ± 0.02	0.73 ± 0.02	0.85 ± 0.03	16 vs 6, 11
E	1.03 ± 0.03	0.52 ± 0.01	0.74 ± 0.02	16 vs 6, 11; 6 vs 11
G	8.19 ± 1.01	3.22 ± 0.27	7.04 ± 1.19	11 vs 6, 16
Intraday comparisons	G vs V, E	G vs V, E	G vs V, E	
Serum insulin post clamp (ng/ml)				
V	2.15 ± 0.55	7.45 ± 1.97	11.54 ± 3.78	16 vs 6, 11; 6 vs 11
E	5.18 ± 1.25	3.85 ± 0.78	1.99 ± 0.37	6 vs 11, 16; 11 vs l6
G	12.95 ± 1.34	7.60 ± 1.26	10.25 ± 1.43	11 vs 6, 16
Intraday comparisons	G vsV, E; V vs E	E vs V, G	E vs V, G	

Values are mean ± S.E.M. for 5 animals. Only significant differences are shown.

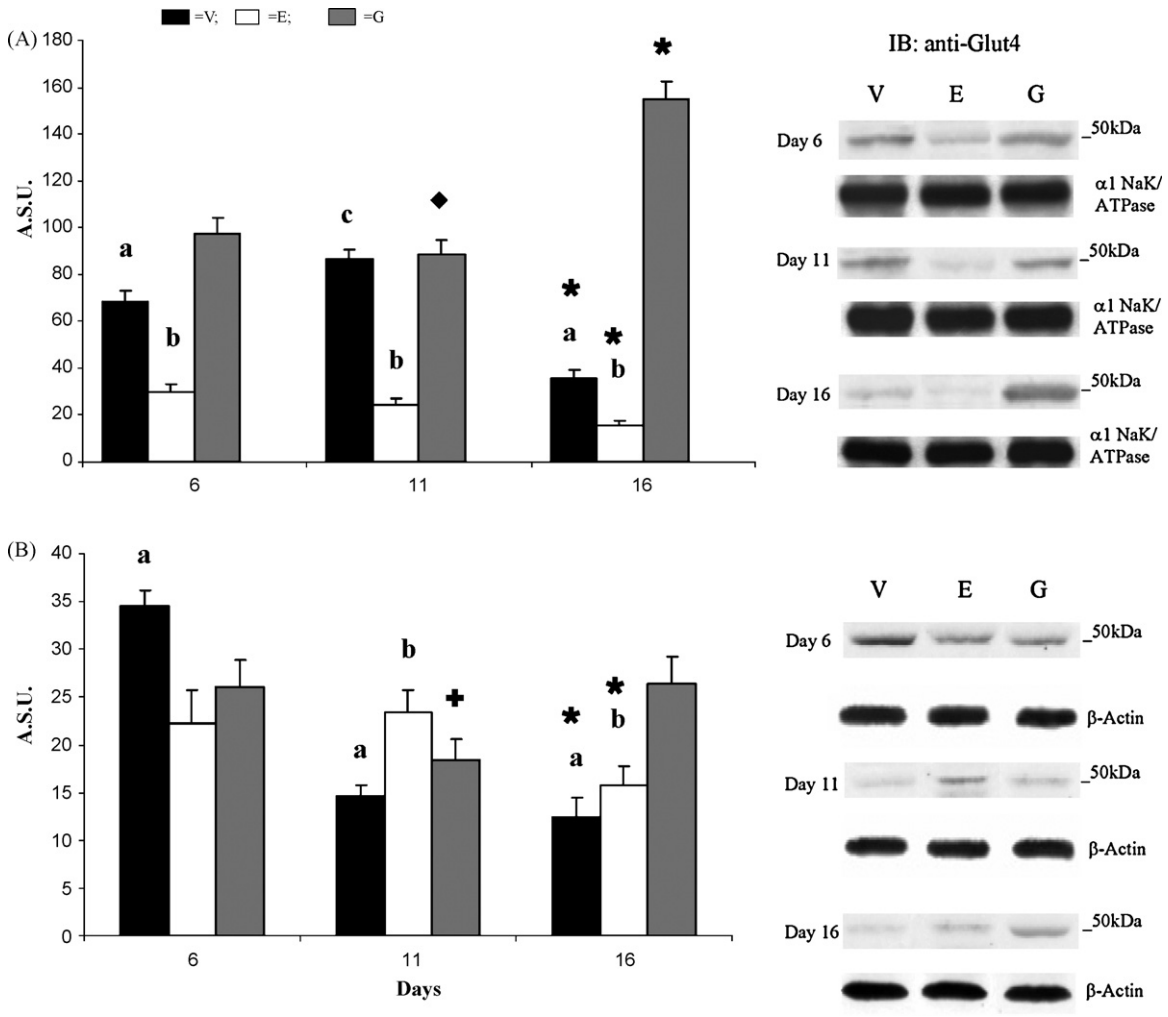

Fig. 3. Changes in Glut4 plasma membrane and total amount of Glut4 ((A) and (B) respectively) in skeletal muscle during pregnancy and 17β-estradiol treatment. Pregnant rats (G), ovariectomized rats treated with vehicle (V) and ovariectomized rats treated with estradiol (E). Scanning densitometry was performed on five experiments. Data are expressed as a mean ± S.E.M. for 5 animals. Only significant differences are shown. A.S.U. = Arbitrary Scanning Units. Intraday comparisons: a = V vs E, G, b = E vs G, c = E vs V; interday comparisons: ✱ = 16 vs 6, 11; ♦ = 11 vs 6; ✚ = 11 vs 6, 16.

presents a comparison of the glucose infusion rate (GUR) in experimental animals. A significant increase in GUR between days 6 and 11 and a significant decrease between days 11 and 16 of pregnancy were observed in the G group. These results highlight the high level of insulin sensitivity in the first half of pregnancy and the decrease in sensitivity during the second half. In studying the effects of estradiol treatment, we observed that in the E group GUR also decreased between days 11 and 16 of the experiment. In the untreated group (V), insulin sensitivity did not change between days 6 and 11 but decreased significantly by the end of experiment (day 16). Intragroup comparisons showed that at days 6 and 16 of the experiment GUR was significantly lower in the V group than in the G and E groups, while there were no differences in the GUR between the E and G groups. At day 11, GUR was also lower in the V group than in the G and E groups, and in the E group than in the G group.

3.3. Plasma membrane and total Glut4 immunoblotting

To determinate how pregnancy triggers changes in insulin sensitivity, the insulin signaling pathway in skeletal muscle and adipose tissue was analyzed on different days of the experiment. Total and plasma-membrane Glut4 was analyzed for two reasons: (1) to determine whether pregnancy or 17β-estradiol treatment modified the total contents of Glut4, although other studies were unable to confirm that total Glut4 content exactly reflects insulin sensitivity [35]; (2) because the plasma-membrane content of Glut4 after clamp experiments reflects insulin-stimulated glucose uptake [35,36]. Firstly, Glut4 protein levels were measured in whole cells and in the plasma membrane of skeletal muscle and adipose tissue after euglycemic–hyperinsulinemic clamping. As described in Section 2, previous Western blot experiments of the plasma-membrane fraction confirmed the presence of the α1 subunit of the Na^+–K^+-ATPase, used as a membrane marker. The amount of this marker was similar between experimental groups, suggesting that the recovery of fractionated plasma membranes was qualitatively similar throughout the experiment.

Fig. 3 shows the plasma-membrane and total amount of Glut4 in the skeletal muscle of experimental animals (Fig. 3A and B, respectively). In the G group, plasma-membrane and total Glut4 levels followed the same profile, being lowest at day 11 and increasing significantly at the end of the experimental period (day 16). In the E group, plasma-membrane and total Gut4 levels decreased significantly by the end of the experiment (day 16), as was the case in V rats. Intergroup comparisons showed that plasma-membrane Glut4 was significantly lower in E rats than in either G or V rats on any day of the experiment. Rats of the V group showed the highest levels of Glut4 at day 6, followed by the E group on day 11, and the G group on day 16.

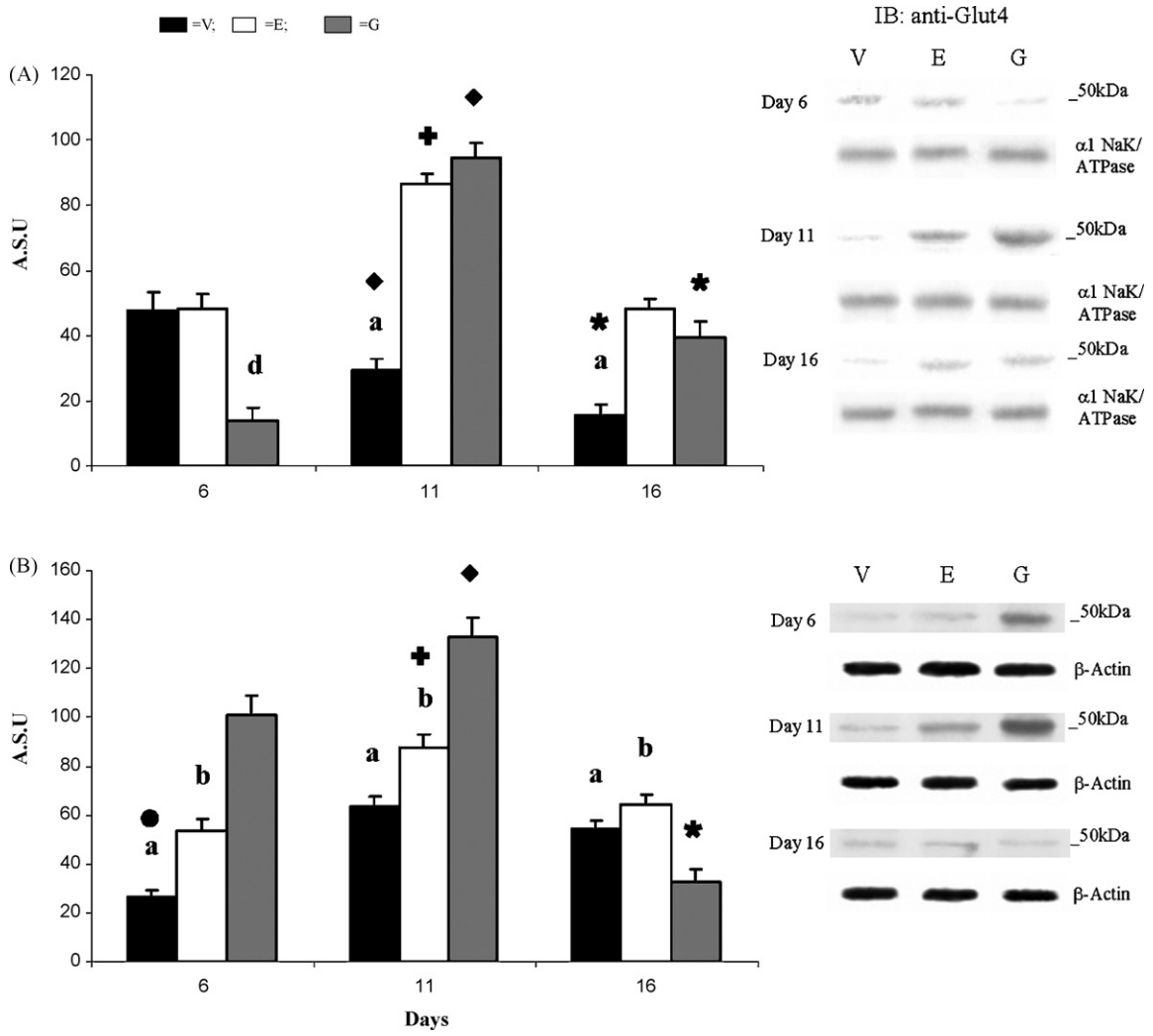

Fig. 4. Changes in Glut4 plasma membrane and total amount of Glut4 ((A) and (B) respectively) in adipose tissue during pregnancy and 17β-estradiol treatment. Pregnant rats (G), ovariectomized rats treated with vehicle (V) and ovariectomized rats treated with estradiol (E). Scanning densitometry was performed on five experiments. Data are expressed as a mean ± S.E.M. for 5 animals. Only significant differences are shown. A.S.U. = Arbitrary Scanning Units. Intraday comparisons: a = V vs E, G, b = E vs G, d = G vs E; interday comparisons: ✱ = 16 vs 6, 11; ♦ = 11 vs 6; ✚ = 11 vs 6, 16; ● = 6 vs 11, 16.

Fig. 4 shows plasma-membrane and total amounts of Glut4 in the adipose tissues of the experimental groups (Fig. 4A and B, respectively). In the G and E groups, plasma-membrane and total Glut4 increased significantly between days 6 and 11, but then decreased significantly between days 11 and 16 of the experiment. In the V group, plasma-membrane Glut4 decreased significantly over the course of the experiment, whereas total Glut4 increased between days 6 and 11 and did not change until the end of treatment. Intergroup comparisons showed that plasma Glut4 at day 6 was lowest in the G group, while this group had the highest amount on day 11. Plasma-membrane Glut4 was higher in E than in V and G animals at day 16. Total Glut 4 was higher in G than in V or E animals at days 6 and 11 and reached its lowest level in the G group at day 16.

3.4. Redistribution of p85α to IR and away from IRS-1

Fig. 5 shows the redistribution of p85α to the IR and away from IRS-1 in the skeletal muscle of the experimental animals. The total amount of p85α decreased in the G and V groups at the end of the experiment, whereas in the E group the opposite occurred (Fig. 5A). Intergroup comparisons showed that p85α levels were significantly higher in the G group than in the E and V groups on any day of the experiment.

The association between IRS-1 and the p85α subunit was detected in IRS-1 immunoprecipitates. In the G and V groups, this interaction increased significantly between days 6 and 11 and decreased between days 11 and 16. In the E group, a decrease at the end of the experimental period was observed; however, there were no changes between days 6 and 11. Intergroup comparisons showed that this interaction was lower in the V group than in the E and G groups at days 6 and 11 and highest in the G group at day 16.

Fig. 5C shows the pool of p85α associated with IR. In the G group, this interaction did not change throughout the experiment. In the E group, there was a significant decrease in the p85α-IR association by the end of the experiment (day 16), while in the V group this association decreased between days 6 and 11 and increased between days 6 and 16. As shown in the intergroup comparisons, at days 6 and 16 the association between p85α and IR was highest in the V group, while on day 11 this interaction was higher in the G group than in the V and E groups.

The redistribution of p85α to the IR and away from the IRS-1 in the adipose tissues of experimental animals is shown in Fig. 6. In rats of the G group, the total amount of p85α decreased signif-

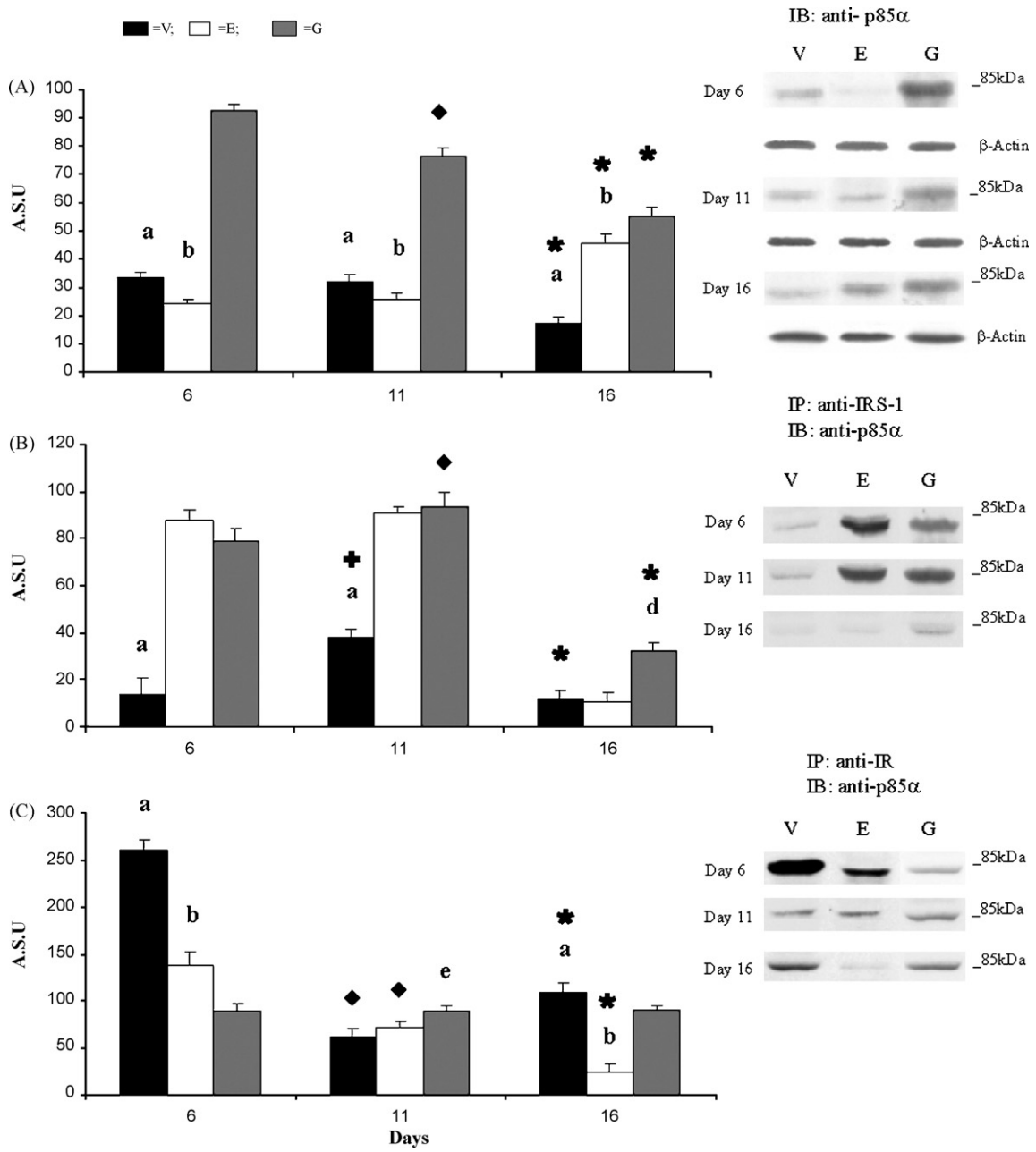

Fig. 5. Changes in p85α redistribution to the insulin receptor away from IRS-1 in skeletal muscle during pregnancy and 17β-estradiol treatment. Pregnant rats (G), ovariectomized rats treated with vehicle (V) and ovariectomized rats treated with estradiol (E). Scanning densitometry was performed on five experiments. Values are means ± S.E.M. for 5 animals. Only significant differences are shown. Intraday comparisons: a = V vs E, G, b = E vs G, d = G vs V, E, e = V vs G; interday comparisons: ✱ = 16 vs 6, 11; ♦ = 11 vs 6; ✚ = 11 vs 6, 16.

icantly throughout the experiment (Fig. 6A). In the E group, p85α levels increased between days 6 and 11 and decreased between days 11 and 16; in the V group the levels increased between days 6 and 11 and did not change until the end of experiment. Intergroup comparisons showed significantly higher p85α levels in the G group than in the E and V groups at days 6 and 16, while the E group had the highest levels at day 11.

The association IRS-1-p85α was confirmed by IRS-1 immunoprecipitates (Fig. 6B). In the G group, there was a significant increase in this association between days 6 and 11 and a significant decrease between days 11 and 16. In the E group, the association increased between days 6 and 11 and remained unchanged until the end of the experiment. Finally, in the V group, the interaction between p85α and IRS-1 increased progressively throughout the experiment. Intergroup comparisons showed that this association was higher in the G group than in the E and V groups at days 6 and 11, whereas the opposite was true at day 16.

The pool of p85α associated with the IR is shown in Fig. 6C. In the G group, this association decreased significantly between days 6 and 11 and increased between days 11 and 16. In the E group, p85α-IR levels increased between days 6 and 11 and did not change until the end of the experiment. In the V group, this association decreased between days 6 and 11 and increased between days 11 and 16. Intragroup comparisons showed that the p85α-IR association was higher in the G group on any day of the experiment.

3.5. Phosphorylation of IRS-1 Ser 612

Phosphorylation of IRS-1 on serine 612 has been shown to impair intracellular signaling by decreasing PI3 kinase activity and Glut4

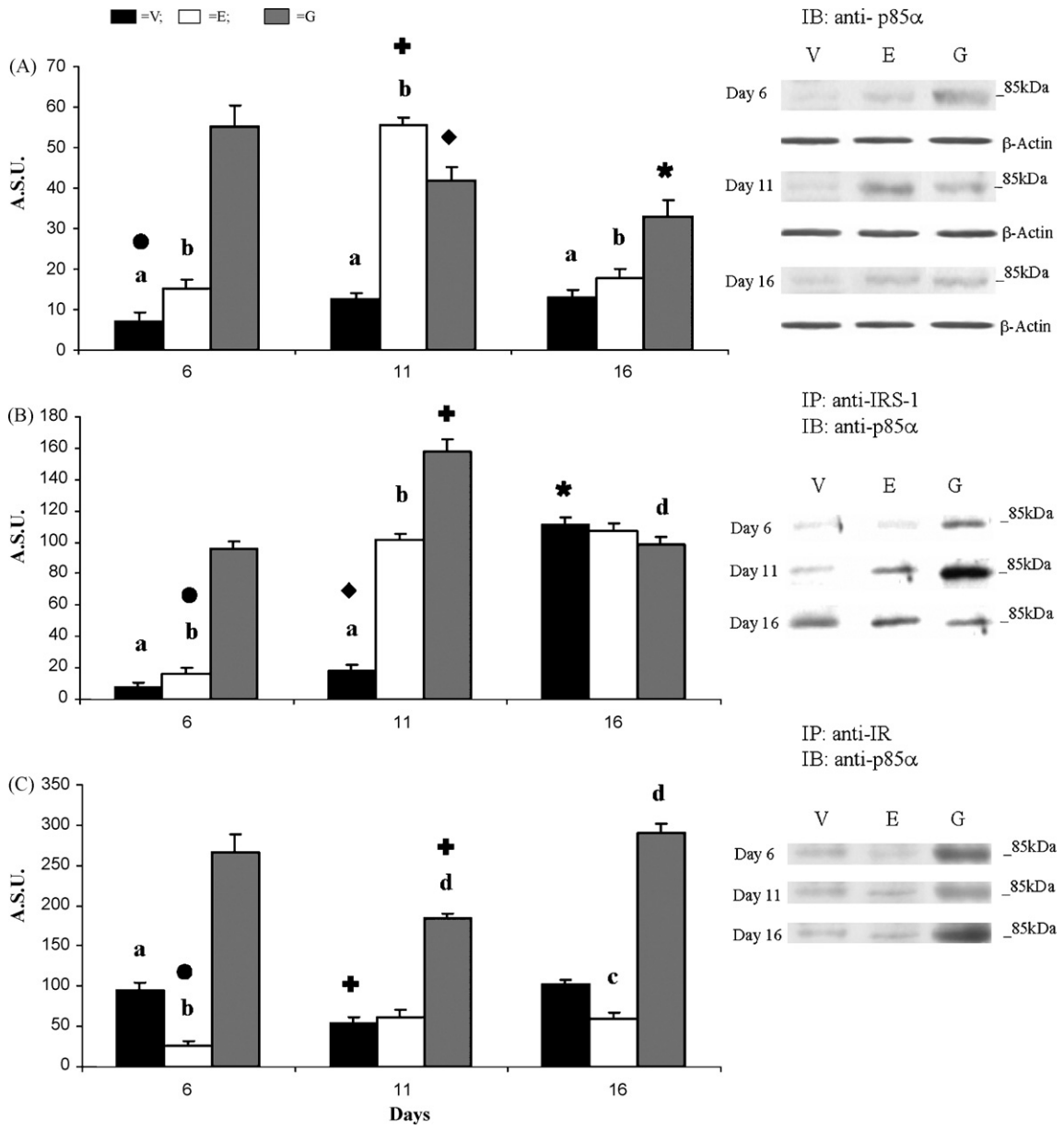

Fig. 6. Changes in p85α redistribution to the insulin receptor away from IRS-1 in adipose tissue during pregnancy and 17β-estradiol treatment. Pregnant rats (G), ovariectomized rats treated with vehicle (V) and ovariectomized rats treated with estradiol (E). Scanning densitometry was performed on five experiments. Values are means ± S.E.M. for 5 animals. Only significant differences are shown. Intraday comparisons: a = V vs E, G, b = E vs G, c = E vs V, d = G vs V, E; interday comparisons: ✱ = 16 vs 6, 11; ♦ = 11 vs 6; ✚ = 11 vs 6, 16; ● = 6 vs 11, 16.

translocation. Since the above results suggested that changes in the insulin signaling pathway occurred throughout the experimental period, we investigated whether these changes were due to alterations in the IRS-1 phosphorylation pattern. As seen in Fig. 7, IRS-1 phosphorylates serine 612. In the skeletal muscle of group G animals (Fig. 7A), this phosphorylation increased significantly between days 6 and 11 and decreased between days 11 and 16. In the E and V groups, serine phosphorylation was detected only at day 16. Intragroup comparisons showed that in the G group the highest levels of IRS-1 serine 612 phosphorylation occurred at days 6 and 11, while in the E group the highest levels were reached at day 16. Fig. 7B shows IRS-1 serine 612 phosphorylation in adipose tissue. In the G group, phosphorylation decreased significantly between days 6 and 11 and increased between days 11 and 16, reaching the highest levels at day 16. In the E group, IRS-1 serine 612 phosphorylation increased significantly throughout the experiment, while in the V group no significant differences were detected at any time. Intragroup comparisons showed that serine 612 phosphorylation was higher in the G group than in the V and E groups at days 6 and 11, but higher in the E group at day 16.

4. Discussion

The molecular mechanisms of insulin resistance during pregnancy remain unknown; however, maternal metabolism is able to limit glucose uptake to ensure that an adequate supply of nutrients is shunted to the fetus. As previously shown [9,18] and confirmed in this study, insulin resistance decreases significantly in pregnant rats between days 11 and 16 (Fig. 2), reflecting the fact that insulin sensitivity is higher in early than in late pregnancy. In the first part of this study, we studied insulin signaling in skeletal muscle and retroperitoneal adipose tissue during pregnancy, focusing on p85α and Glut4. Our results showed a tissue-specific adap-

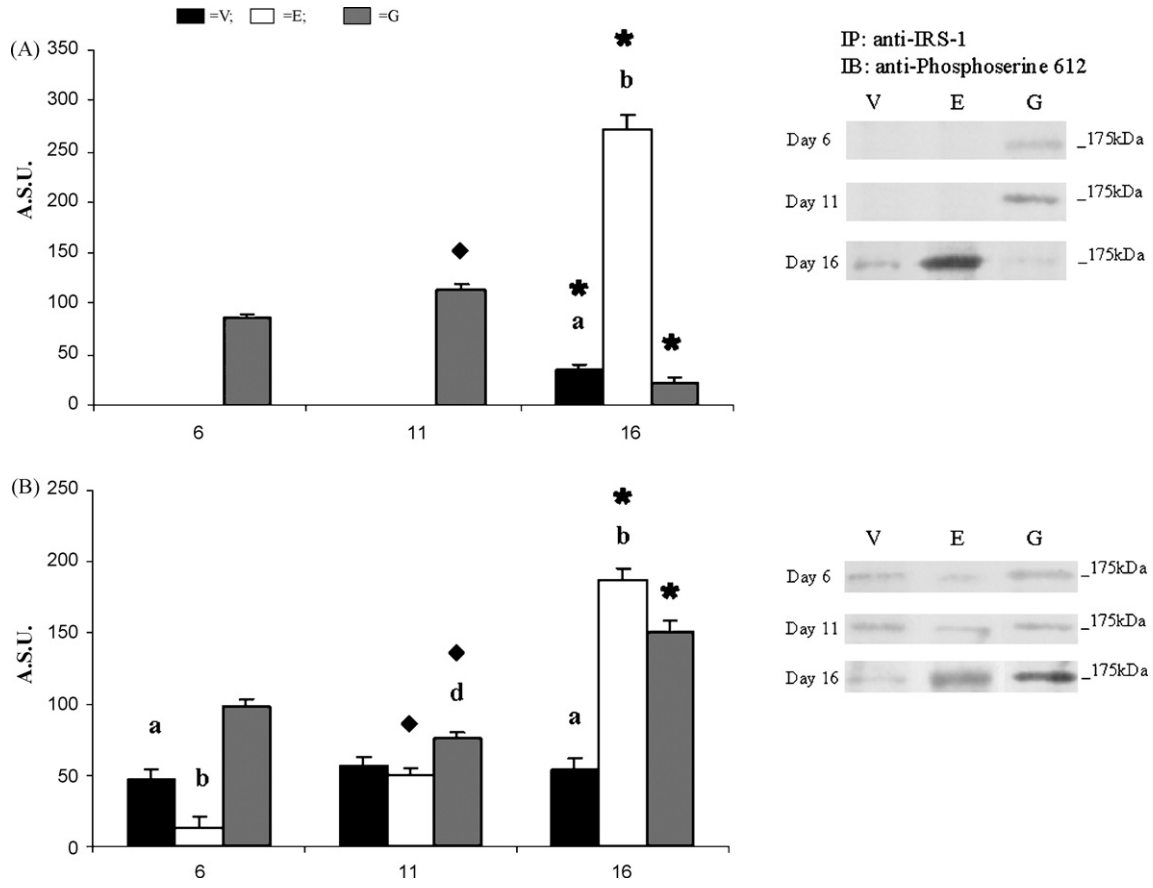

Fig. 7. Changes in serine 612 phosphorylation of IRS-1 in skeletal muscle (A) and adipose tissue (B) during pregnancy and 17β-estradiol treatment. Pregnant rats (G), ovariectomized rats treated with vehicle (V) and ovariectomized rats treated with estradiol (E). Scanning densitometry was performed on five experiments. Values are means ± S.E.M. for 5 animals. Only significant differences are shown. Intraday comparisons: a = V vs E, G, b = E vs G, d = G vs V, E; interday comparisons: ✱ = 16 vs 6, 11; ♦ = 11 vs 6.

tation of insulin signaling; specifically, in retroperitoneal adipose tissue and skeletal muscle, there was a significant decrease in the association between p85α and IRS-1 on days 11–16 of pregnancy (Figs. 5 and 6B). However, while in retroperitoneal adipose tissue the amount of plasma-membrane Glut4 decreased during this same period (Fig. 4A), in skeletal muscle, surprisingly, levels of the protein significantly increased (Fig. 3A). These results implicate adipose tissue in the development of insulin resistance associated with gestation, through modulation of plasma-membrane Glut4. Moreover, we also showed that during late pregnancy there is a decrease in the total content of Glut4 in adipose tissue (Fig. 4B), suggesting a decrease in Glut4 synthesis rather than a defect in Glut4 translocation to the plasma membrane, in agreement with the findings of Yamada et al. [37]. However, an extrapolation of our results in retroperitoneal adipose tissue to white adipose tissue is premature, since Pujol et al. [38] reported regional differences in the metabolic regulation of this tissue throughout pregnancy.

It has been proposed that the p85α subunit of PI3-K binds directly to autophosphorylated IR, thus p85α showing lipid and serine kinase activity [39,40]. In skeletal muscle of mice with gestational diabetes mellitus (GDM), the redistribution of PI3-K to the IR is the mechanism underlying insulin resistance in GDM pregnancies. In the present study, the p85α-IR interaction was examined in skeletal muscle and adipose tissue during pregnancy. In adipose tissue, there was a significant increase in this association between days 11 and 16 (Fig. 6C), accompanied by an increase of IRS-1 serine 612 phosphorylation (Fig. 7B). Sevillano et al. [41] also reported an increase in IRS-1 serine phosphorylation, although they studied Ser 307. IRS-1 has more than 50 potential sites of serine phosphorylation. The phosphorylation of Ser 307 and Ser 612 has in both cases been related to a decrease in insulin sensitivity, but with different repercussions for insulin signaling. Serine 307 is located at the end of the phosphotyrosine binding domain (PTB) and its phosphorylation uncouples IRS-1 from IR, decreasing tyrosine phosphorylation of the latter and thereby its degradation. Serine 612 is located close to the C-terminus and its phosphorylation reduces the PI3-K association (for review, [42]).

However, in skeletal muscle, although there was a slight but significant increase in the p85α-IR interaction (Fig. 5C), it was not linked to an increase in IRS-1 serine 612 phosphorylation (Fig. 7A). This result supports the conclusion that the development of insulin resistance in skeletal muscle through p85α redistribution is limited to complicated pregnancies, such as in GDM. This is in general agreement with Shao et al.'s work [40] but, unfortunately, adipose tissue was not examined in that study.

In insulin-resistant states such as type 2 diabetes [43], an impaired insulin response is observed in adipose tissue before it becomes apparent in skeletal muscle. It is also clear that adipose tissue plays a pivotal role in the development of insulin resistance. Taking these earlier results together with those reported here, it seems that during normal pregnancy retroperitoneal adipose tissue is involved in the development of systemic insulin resistance through a mechanism involving p85α redistribution to the IR. This may well be a physiological situation, because one of the main objectives of pregnancy-related insulin resistance is to mobilize fuel stores from maternal adipose tissue to ensure adequate fetal development. However, adipose tissue is a complex endocrine organ, and each of the different anatomic depots has a specific response dur-

ing pregnancy [32]. Our results suggest that mid-pregnancy (day 11) serves as a "hinge point" between a lipogenic and a lipolytic state due to changes in insulin sensitivity, but specific assays are necessary to confirm this hypothesis. Rodríguez-Cuenca et al. [32] found that the changes observed in adipose tissue during pregnancy are also linked to specific regulation of estrogen receptor expression in adipose tissue. The lipolytic processes typical of late pregnancy would therefore be caused by high plasma estradiol concentrations and low amounts of ERα. Moreover, we and others have described specific interactions between this non-nuclear ERα and proteins involved in insulin signaling, such as IRS-1 and p85α [44,45]. Thus, there may be a direct interaction between insulin-signaling proteins and ERα in peripheral, mainly adipose tissues that modulates adaptations in insulin sensitivity during pregnancy. However, further studies are needed to confirm whether this is indeed the case.

In a series of earlier studies [10,11,29,30], we found that 17β-estradiol plays a very important role in the regulation of insulin sensitivity during pregnancy. Here, we sought to determine whether 17β-estradiol is involved in this process; specifically, in tissue-specific insulin signaling adaptations. To this end, ovariectomized rats were treated for 6, 11, and 16 days with 17β-estradiol in order to simulate gestational plasma levels (Fig. 1). In the E group, although the variations in insulin sensitivity were similar to those characteristic of pregnancy (Fig. 2), the molecular mechanisms responsible for these actions differed in that, in the E group, they seemed to be related to a significant decrease in skeletal muscle and adipose tissue Glut4 (Figs. 3 and 4). By contrast, the estradiol effect on Glut4 was not the same in the two tissues. In skeletal muscle, estradiol seemed to cause a reduction of plasma-membrane Glut4 levels (Fig. 3A) whereas the opposite occurred in adipose tissue (Fig. 4A). Moreover, there was a tissue-specific redistribution of p85α: in skeletal muscle, p85α-IRS-1 and p85α-IR associations had decreased by the end of the experiment (Fig. 5B and C) while in adipose tissue the p85α-IRS-1 interaction increased but the p85α-IR interaction was unchanged by hormonal treatment (Fig. 6B and C). Therefore, in the skeletal muscle and adipose tissue of the estradiol-treated group, p85α redistribution is not likely to be involved in insulin resistance, which instead seems to be more related to a decrease in total and plasma-membrane Glut4 (Figs. 3 and 4). Thus, we can conclude that changes in the gestational 17β-estradiol plasma level alone are not able to reproduce the redistribution of p85α to the IR and away from IRS-1, suggesting that factors present in pregnancy but absent from our experimental design are responsible for this response. For example, it has been reported that, during pregnancy, the increase in plasma progesterone levels is linked to a specific modulation of progesterone receptor expression in adipose tissue [32]. There is also growing evidences that the progesterone receptor is localized in non-nuclear compartments [46]. To our best knowledge, a possible interaction between insulin signaling proteins and the progesterone receptor has not been studied. Accordingly, we cannot rule out that during pregnancy specific cross-talk between non-nuclear estrogen and progesterone receptors and insulin signaling pathways results in maternal metabolic adaptations ensuring fetal development. Moreover, recently reported findings strongly suggest that white adipose tissue secretes several bioactive peptides, collectively referred to as adipokines. Although their physiological functions are mostly unknown, some of them are known to be involved in glucose homeostasis. It was therefore suggested that insulin resistance in adipose tissue during pregnancy leads to important changes in adipokine expression that may be relevant in the mechanisms underlying insulin resistance (for review, [47–49]). For example, resistin mRNA expression in white adipose tissue is significantly higher in early than in late pregnancy, although serum increases of this adipokine during late pregnancy remain elevated until lactation [50].

In summary, retroperitoneal adipose tissue contributes to the development of systemic insulin resistance during late pregnancy by means of a specific redistribution of the p85α pool to the IR and away from IRS-1 and by an increase in IRS-1-serine 612 phosphorylation. In addition, while 17β-estradiol by itself is unable to reproduce these gestational-related-changes, it was implicated in the redistribution of p85α in a time- and tissue-dependent manner. More studies are needed to understand the interaction between estrogen and insulin sensitivity, focusing on a direct interaction between the non-nuclear estrogen receptor and key insulin-signaling proteins in order to understand the maternal adaptations that promote successful fetal development.

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