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Effect of Treatment with Different Doses of 17-b-Estradiol on Insulin Receptor Substrate-1.

Celestino González, Ana Alonso, Natalia A Grueso, Fernando Díaz, Manuel M Esteban, Serafina Fernández, Angeles M Patterson

Department of Functional Biology, Physiology Area, University of Oviedo, Oviedo, Spain.

ABSTRACT

Context Ovarian hormones modulate insulin sensitivity, but their exact role remains unclear.
Objective We tried to determine whether different doses of 17-β-estradiol cause changes in the regulation of insulin receptor substrate (IRS-1) levels, and if so, the possible implications in insulin sensitivity.
Design Ovariectomized rats were treated with different doses of 17-β-estradiol at 6, 11 and 16 days.
Main outcome measures Immunoprecipitation and Western blotting for IRS-1 were performed in different tissues.
Results We found that estradiol treatment has an influence on the amount of IRS-1 but that it acts in different ways depending on the tissue studied, on the length of treatment, and on the doses employed.
Conclusions Our results suggest that low concentrations of 17-β-estradiol could be responsible for the upregulation of insulin receptor substrate 1, increasing insulin sensitivity in muscle and adipose tissue. However, insulin receptor substrate 1 is downregulated with high concentrations of 17-β-estradiol, thus these high hormone plasma levels could favour insulin resistance in peripheral tissues. The role of 17-β-estradiol seems to modulate insulin receptor substrate 1 levels in insulin dependent tissues, but in a different manner in each tissue. These novel findings are important for improving knowledge about the possible risk for insulin resistance in women taking oral contraceptives or receiving hormone replacement therapy at menopause.

INTRODUCTION

Various clinical observations and experimental data suggest that estrogen and progesterone can modulate insulin sensitivity in females [1, 2]. In this sense, clinical conditions such as pregnancy, where estrogen and progesterone concentrations are markedly elevated, have a substantial effect on carbohydrate metabolism as well as on the alteration of insulin sensitivity [3]. Moreover, in women taking the combined oral contraceptive pill, artificially increased levels of the sex steroid hormones estrogen and progesterone were found to affect glucose tolerance and insulin sensitivity [4, 5]. However, it remains unclear whether estrogens alone or progestins alone can cause insulin resistance, or whether it is only a combination of both which produces this effect.
Insulin action begins upon its binding to its cell surface receptor [6, 7]. The discovery of tyrosine kinase activity in the insulin receptor

implies that the mechanism of insulin action involves the tyrosine phosphorylation of intracellular substrates [7, 8], thereby transmitting the insulin signal. Insulin receptor substrate 1 (IRS-1) is considered the major substrate for the insulin receptor, and contains motifs that, after tyrosine phosphorylation, are binding sites for proteins containing Src homology (SH2) domains [9]. The association of some proteins (phosphatidylinositol 3´-kinase: PI3-K, growth factor receptor-bound protein 2: Grb2, and tyrosine phosphatase SHP2) with IRS-1 permits their activation and the transmission of the insulin signal [10]. Thus, IRS proteins amplify the insulin receptor signal by eliminating the stoichiometric constraints encountered by receptors that directly recruit proteins containing SH2 site domains to their autophosphorylation sites.

Molloy *et al*. [11] and Lee *et al.* [12] showed that estrogen induces the expression of the downstream signaling molecules, IRS-1 and IRS-2. Estrogen induction of IRS-1 expression was associated with increased tyrosine phosphorylation of IRS-1 and correlated with enhanced downstream mitogen-activated protein kinase (MAPK) activation. While the increase in IRS-1 expression generally mirrored the increase in tyrosine phosphorylation, the authors could not rule out the possibility that the increase in tyrosine phosphorylation of IRS-1 results from a change in stoichiometry or the sites of phosphorylation. Furthermore, there may be other factors controlling the phosphorylation of IRS-1. For instance, decreased phosphorylation of IRS-1 may be mediated by the antiestrogen-induction of a specific tyrosine phosphatase activity [13, 14].

Several lines of evidence indicate that phosphorylation of IRS-1 on serine/threonine residue has an inhibitory effect on insulin signaling [15, 16, 17]. Following the increase in IRS-1 serine/threonine phosphorylation, the ability of insulin to phosphorylate IRS-1 on tyrosine residues is decreased. It can be concluded, therefore, that a modulated pattern of phosphorylation may also play a role in its reduced response to insulin in the insulin-resistant state [18].

On the other hand, the possible contribution of estradiol to the degradation pathway of IRS-1 is not sufficiently known, but the identification of well-defined intermediates suggests that there may be a specific degradation pathway. Since the role of IRS-1 is to act as a docking protein which assembles a signaling complex, IRS-1 degradation may be an important way of regulating IRS-1 signaling.

In this preliminary study, we tried to demonstrate whether different doses of 17-β-estradiol could influence the regulation of the amount of IRS-1 and the possible implication of this hormone in insulin sensitivity during gestation. Therefore, this study was designed to test whether the amount of IRS-1 in the liver, skeletal muscle and adipose tissue could be modified by the concentration of 17-β-estradiol and/or by the length of exposure to the hormone.

MATERIAL AND METHODS

Animals

Twelve-week-old virgin female Wistar rats (from the Biotery of the University of Oviedo) weighing 250-280 g, and kept at standard conditions of temperature (23±3 ºC) and humidity (65±1%), with a regular lighting schedule of a 12 h light/dark cycle (08:00 am - 08:00 pm) were used. The animals were fed a standard diet (Panlab A04, Barcelona, Spain) and had free access to water. All experimental manipulations were performed between 09:30 am and 12:30 am.

Experimental Design

Three days before initiating the hormonal treatment (day -7), the rats were ovariectomized through a midline incision using light ether anaesthesia. The ovariectomized rats were

randomly separated into four groups: control (V), estradiol (E), estradiol x 10 (EX10) and estradiol x 100 (EX100) and housed individually throughout the experiment.

After surgery, the ovariectomized rats were allowed 3 days to recover from the stress of surgery and decrease their hormonal levels. From day -4, the rats were injected subcutaneously every twelve hours (09.00 am and 09:00 pm) for 20 days with 0.1 ml of a 17-β-estradiol (SIGMA Chemical Co., San Louis, USA) suspension in olive oil/ethanol (3:2 v/v). The control group (V) injected with the vehicle (olive oil/ethanol 3:2 v/v) was followed in parallel. In the E group, different doses of 17-β-estradiol were injected in order to simulate the plasma levels that we observed in normal pregnant rats [19, 20]. In the EX10 group, the doses of 17-β-estradiol injected were ten times E and in the EX100 group, the doses of 17-β-estradiol injected were one hundred times E.

The hormonal treatment was applied according to the temporal diagram reported in Table 1.

Table 1. Temporal diagram of the 17-β-estradiol doses.

Day	17-b-estradiol dose (E)	Note
-7	-	Ovariectomy
-6	-	
-5	-	
-4	0.326 µg/day	
-3	0.326 µg/day	
-2	0.326 µg/day	
-1	0.326 µg/day	
0	0.326 µg/day	
1	0.326 µg/day	
2	0.326 µg/day	
3	0.326 µg/day	
4	0.326 µg/day	
5	0.326 µg/day	
6	0.163 µg/day	Day animals were sacrificed
7	0.163 µg/day	
8	0.163 µg/day	
9	0.163 µg/day	
10	0.163 µg/day	
11	0.326 µg/day	Day animals were sacrificed
12	0.326 µg/day	
13	0.326 µg/day	
14	0.326 µg/day	
15	0.326 µg/day	
16	-	Day animals were sacrificed

Animals were sacrificed randomly on the 6th, 11th and 16th days (6 animals/subgroup). These days were selected as changes were found in the sensitivity to insulin action during pregnancy in the Wistar rat [19].

On the day of sacrifice and after 12 h of fasting on days 6, 11 and 16, the animals were anesthetized with 3.3 mL/kg body weight of intraperitoneal ekytesin (0.96 g/mL sodium pentobarbital, 4.02 g/mL chloral hydrate, 2.12 g/mL magnesium sulphate, 40% propilenglicol, 10% ethanol). As soon as anaesthesia was assured by the loss of pedal and corneal reflexes, a blood sample (1 mL) was collected from the jugular vein in heparinized tubes, centrifuged at 3000 rpm during 20 min at 4 ºC and plasma was immediately drawn off and stored frozen at –20 ºC until assayed. Plasma 17-β-estradiol was measured by RIA using Immuchen kits of cover tubes (ICN Biomedicals Inc., Costa Mesa, USA). The assay sensitivity was 10 pg/mL, and the intra-assay coefficient of variation was 12.26%. All samples were measured on the same day. Each sample was assayed in duplicate.

Finally, samples of different tissues (liver; skeletal muscle: *flexor digitorum superficialis, extensor digitorum longus, soleus* and *extensor digitorum lateralis*; retroperitoneal adipose tissue) were collected and immediately frozen in liquid nitrogen for future experiments and the animals were killed by bleeding.

Immunoprecipitation and Western Blotting

The samples of liver, skeletal muscle and adipose tissue were washed with ice-cold sterile phosphate-buffered saline (PBS) and homogenized immediately in 3 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet® P40 (Roche Diag, Barcelona, Spain), 0.05% sodium deoxycholate, 0.1% ortovanadate 1 M) at 4º C using a Polytron® PT-MR-3000 (Kinematica AG, Lucern, Switzerland) operated at maximum

speed for 30 sec. The extracts were centrifuged at 12,000 *g* at 4º C for 10 min in order to remove insoluble material. After centrifugation, the protein content was measured by the Bradford dye-binding method [21] using the Bio-Rad (Hercules, USA) reagents and BSA as standard. The aqueous fraction containing 250 µg of protein for liver and muscle and 150 µg for adipose tissue were used for immunoprecipitation (IP) with 0.25 µg of polyclonal antibody against the insulin receptor substrate 1 (IRS-1) (sc-559-G, Santa Cruz Biotech, Inc., Santa Cruz, USA). The immune-complexes were precipitated with protein G-agarose beads (Roche Diag., Barcelona, Spain) overnight at 4º C in a rocking platform and 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). 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.

For total extraction, similar size aliquots were subjected to SDS-PAGE (7% Tris-Acri-Bis) in a miniature slab gel apparatus (Bio-Rad, Hercules, USA). The prestained molecular-mass standards used were myosin (218 kDa), β-galactosidase (126 kDa), bovine serum albumin (90 kDa), carbonic anhydrase (43.5 kDa), soybean trypsin inhibitor (33.9 kDa), lysozyme (17.4 kDa) and aprotinin (7.6 kDa) (Bio-Rad, Hercules, USA). Electrotransfer of proteins from the gel to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia Biotech, Barcelona, Spain) was performed for 60 min at 50 V (constant) in a miniature transfer apparatus (Mini-Protean, Bio-Rad) as described by Towbin *et al.* [22].

Non-specific protein binding to the nitrocellulose membranes was reduced by preincubating the filter for two hours at room temperature in blocking buffer (TNT, 7% BSA) and Western blot analysis was performed (Western-Light, Chemiluminescent Detection System, TROPIX Inc., Bedford, USA) using a 1:2,000 dilution of polyclonal antibody against the IRS-1 as the primary antibody, followed by alkaline-phosphatase-conjugated anti-rabbit IgG antibody (TROPIX Inc., Bedford, USA) for detection. Finally, the membranes were rinsed several times with blocking buffer without BSA and proteins were detected with the ECL reagent (Amersham Pharmacia Biotech, Barcelona, Spain) according to the manufacturer's instructions and using autoluminography on Kodak X-Omat film reagent (Amersham Pharmacia Biotech, Barcelona, Spain).

Western blots were quantified using a digital scanner (AX-110, Nikon, Surrey, UK) and NIH Image 1.57 software (Scion Co., Maryland, USA).

ETHICS

The experiments were carried out in accordance with the rules of laboratory animal care.

STATISTICS

Data are expressed as mean ± SEM. We used analysis of variance followed by a Tukey test in the statistical analysis of 17-β-estradiol plasma levels. Since the distribution of the IRS-1 levels in different tissues was skewed, the Mann-Whitney U -Wilcoxon Rank Sun W test was used. A P value less than 0.05 was considered significant. Statistical analysis was performed using SPSS for Windows v.6.01.

RESULTS

Plasma Levels of 17-b-Estradiol

The plasma levels of 17-β-estradiol are shown in Figure 1. The results obtained in the E group were similar to normal pregnant rats at 5, 10 and 15 days of pregnancy [19, 20]. In this group, we found similar values at 6 and 11 days

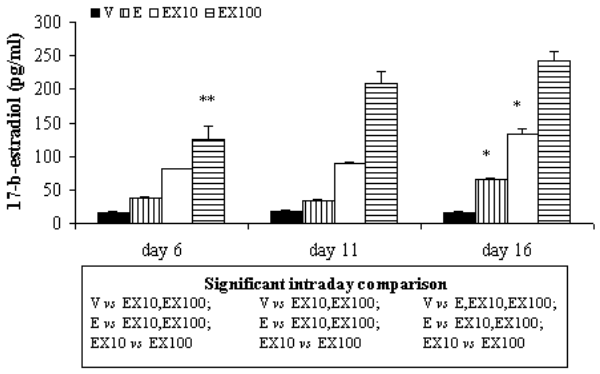


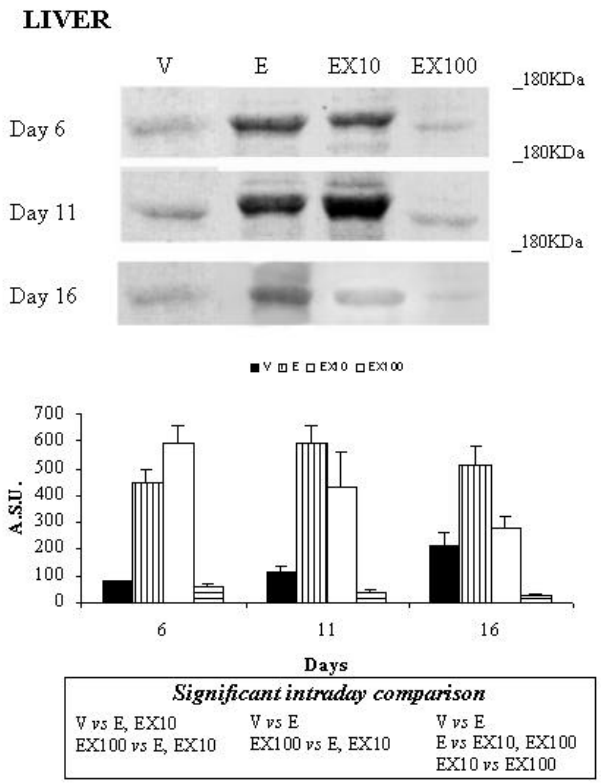
Figure 1. 17-β-estradiol plasma levels in ovariectomized rats (V) and rats treated with different doses of 17-β-estradiol (E, EX10, EX100). Data are expressed as mean ± SEM. Only significant differences are shown.
Significant interday comparison: * day 16 *vs.* days 6 and 11; ** day 6 *vs.* days 11 and 16

of treatment and a significant increase was observed at day 16 *vs.* days 6 and 11. The results obtained in the EX10 and the EX100 groups were significantly higher than E. However, the profile in both groups was parallel with the E group. The 17-β-estradiol plasma levels were dependent on the solution injected. Thus we observed that the plasma concentration of 17-β-estradiol increases: at day 6 (238% in E *vs.* V, 509% in EX10 *vs.* V and 787% in EX100 *vs.* V), at day 11 (188% in E *vs.* V, 488% in EX10 *vs.* V and 1,145% in EX100 *vs.* V) and at day 16 (401% in E *vs.* V, 803% in EX10 *vs.* V and 1,463% in EX100 *vs.* V).

Protein Content of IRS-1

Figure 2 shows a representative experiment in which the solubilized liver, skeletal muscle and adipose material were subjected to immunoprecipitation using an anti-IRS-1 antibody. After SDS-PAGE and electrotransfer, nitrocellulose membranes were incubated in the presence of anti-IRS-1 antibody. In the immunoprecipitated materials, ECL detection showed one band of 126-218 kDa corresponding to IRS-1.

In the liver, at day 6 of experimentation we observed a significant increase in the IRS-1 level in the E and the EX10 groups as compared to the V and the EX100 groups. The same result was observed at day 11 but the difference between the EX10 and the V groups did not reach the level of significance. At day 16, we found a significant increase in the E group as compared to the other groups and a significant decrease in the EX100 group as compared to the EX10 group; no significant differences were found between the V and the EX10 groups. The length of treatment does not significantly change the amount of IRS-1, but we observed a remarkable progressive increase in the V group from day 6 to day 16 and a remarkable decrease in the EX10 and the EX100 groups in the same period.
The level of IRS-1 in skeletal muscle was significantly increased in the E group as compared to the other groups at day 6. However, at day 11 we found a significant increase in the V group as compared to the other groups; we also showed that the EX100 group had significantly higher IRS-1 levels than the E and the EX10 groups and these levels were significantly higher in the E group as compared to the EX10 group. Only significant differences were found between V and EX10 groups at day 16. As far as the comparisons between the different days were concerned, we observed a significant increase of IRS-1 levels in the V group from day 6 to day 11 and a significant decrease from day 11 to day 16. In the R group, the IRS-1 levels were significantly decreased at days 11 and 16 *vs.* day 6, while the length of treatment significantly increased these levels in the EX10 (day 16 *vs.* days 6 and 11) and the EX100 (days 11 and 16 *vs.* day 6) groups.
In adipose tissue, the level of IRS-1 was found to be significantly higher in the E and the EX10 groups as compared to the V group at day 6 of treatment. However, this tendency changes at days 11 and 16 of treatment: the level of IRS-1 was significantly lower in the EX100 as



compared to the other groups at days 11 and 16. We also observed that the tendency of the IRS-1 levels throughout treatment was different depending on the timing and the 17-β-estradiol dose. In the V group, the level of IRS-1 rose between days 6 and 16, whereas in the EX10 and the EX100 groups, the opposite occurred: this level decreased at day 16 *vs*. days 6 and 11 in the EX10 group, as well as progressively decreasing during the treatment period in the EX100 group. However, in the E group we found a significant decrease between days 6 and 11 and a significant increase between days 11 and 16.

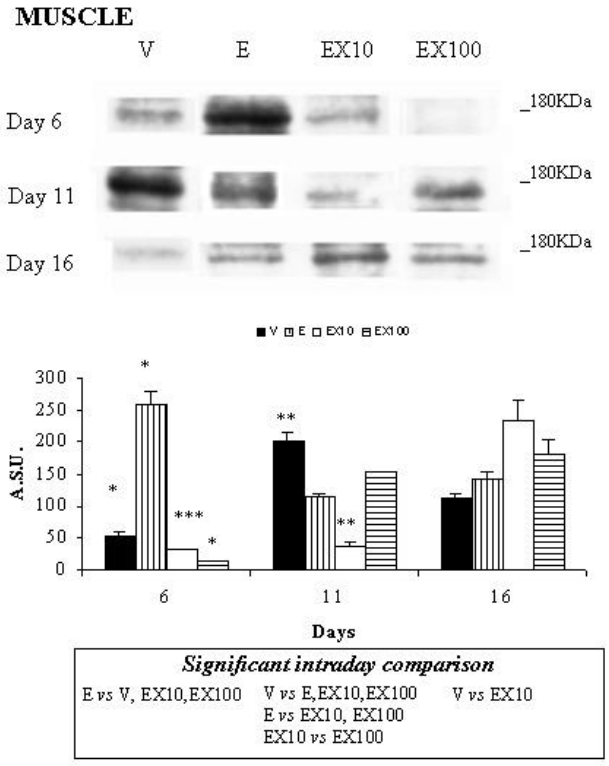


Figure 2. IRS-1 levels in liver, skeletal muscle and adipose tissue in ovariectomized rats treated with different doses of 17-β-estradiol. The proteins were isolated, immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-IRS-1 antibody. Scanning densitometry was performed for five independent experiments. Data are expressed as mean ± SEM. Only significant differences are shown.
Significant interday comparison: * day 6 *vs.* days 11 and 16; ** day 11 *vs*. day 16; *** day 6 *vs.* day 16
A.S.U.: Arbitrary scanning units

DISCUSSION

We have recently showed that 17-β-estradiol could be responsible for the increase in insulin

sensitivity during early pregnancy when the plasma concentrations of 17-β-estradiol and progesterone are low [23]. During late pregnancy when the plasma concentrations of both hormones are high, the role of 17-β-estradiol could be that of antagonizing the effect of progesterone by diminishing insulin sensitivity. We therefore propose that some of these adaptative changes in carbohydrate metabolism during pregnancy can be focused on the IRS-1.

The novel finding of this study is that 17-β-estradiol seems to be responsible for controlling insulin sensitivity in females throughout gestation. In this sense, the present results confirm those of a previous study [23], namely, when the plasma concentration of 17-β-estradiol is similar to that found in early pregnancy, IRS-1 is upregulated and the insulin sensitivity increases in the peripheral tissues (skeletal muscle and adipose). However, IRS-1 is downregulated when the plasma concentration of this hormone is similar to that found in late pregnancy, diminishing the insulin sensitivity in peripheral tissues. We suggest, in accordance with Lee *et al.* [12], that a low concentration of 17-β-estradiol increases IRS-1 expression by a transcriptional mechanism because the IRS-1 promoter does have four consensus half-estrogen response elements [24]. However, when the concentration of 17-β-estradiol is high, the binding of many complexes of estradiol receptor-estradiol to the IRS-1 promoter could induce a decrease in IRS-1 expression, probably by displacement of other transcription factors linked to the IRS-1 promoter. Among these factors, we could point out progesterone, lactogenic hormones, growth hormone, etc, [1, 2, 3, 23, 25, 26].

In the present study, in the E group, the tissues studied were found to perform differently. In this way, there are no significant changes in the liver IRS-1 level throughout hormone treatment, but, in skeletal muscle and adipose tissue, a significant decrease was observed from day 6 to day 11 and from day 6 to day 16. These findings confirm a previous study which demonstrated that gestation could be divided into two periods, namely, the period of early gestation, which is characterized by an increase in sensitivity to insulin action in the maternal tissues and the period of late gestation, characterized by a decrease in this sensitivity [19]. In the light of the present results our hypothesis is that, during early pregnancy, the peripheral "insulin sensitivity" (larger amount of IRS-1) together with high insulin plasma levels (data not shown) could favour the storage of energetic reserve in the adipose tissue, whereas during later pregnancy, these adaptative changes are reversed. The decrease in insulin plasma levels (data not shown) together with less insulin sensitivity in adipose tissue facilitate the lipolysis and the increase in triglycerides plasma levels which can be observed at the end of pregnancy [20].

In relation to the EX10 and the EX100 groups, we can note that the higher doses of 17-β-estradiol (EX100 group) significantly decreased the amount of IRS-1 with respect to the E group in the liver and adipose tissue. However, in skeletal muscle, this fact only occurs at day 6 of treatment. The length of exposure to the hormone action seems to amplify the decrease in IRS-1 levels in the EX100 group, also with the exception of skeletal muscle. Similar results were observed in the EX10 group. These findings seem to demonstrate that liver and adipose tissue are the tissues most affected by 17-β-estradiol action with respect to the amount of IRS-1, and that the effect of this hormone was dose and timing dependent in agreement with other authors postulating that the effect of estrogen therapy on insulin and glucose metabolism depend on the type of estrogen, dose, length of treatment, etc. [4, 27, 28]. The roles of liver and adipose tissue are very important during pregnancy, because the liver is the most important tissue for insulin clearance [29] and adipose tissue is the most important energetic reserve. In this sense, our hypothesis, in the light of the present results, is

that during early pregnancy, the liver could be discouraging insulin clearance, so the high insulin plasma levels (data not shown) together with high sensitivity in adipose tissue could favour the storage of energetic reserve. However, in late pregnancy, the role of the liver could be to increase insulin clearance, so the decrease in insulin plasma levels (data not shown) together with less insulin sensitivity in adipose tissue facilitate the lypolisis and the increase in triglycerides plasma levels which can be observed at the end of pregnancy [20]. We consider that these novel findings are very important for a better understanding of the role of estrogen during pregnancy. Moreover, these results also illustrate the great importance of estrogen dosage and concentration as regards glucose metabolism in hormonal replacement therapy in women at menopause and in women taking oral contraceptives.

In summary, our present findings suggest that low concentrations of 17-β-estradiol similar to early pregnancy levels could be responsible for the upregulation of IRS-1, increasing insulin sensitivity in peripheral tissues (muscle and adipose). However, this protein is downregulated with high concentrations of 17-β-estradiol similar to late pregnancy, thus these high hormone plasma levels could favour insulin resistance in the peripheral tissues. Consequently, the role of 17-β-estradiol seems to be to modulate the amount of IRS-1 in insulin dependent tissues, but in a different manner in each tissue. In spite of this, the role of this hormone could appear to be slightly altered in the presence of high plasma concentrations of progesterone, lactogenic hormones and growth hormone, just as occurs during normal pregnancy. Moreover, we think that these novel findings are very important in order to improve knowledge about the possible risk for insulin resistance in women taking oral contraceptives or hormone replacement therapy at menopause.

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Key words Insulin; Insulin Resistance; Rats

Abbreviations Grb2: growth factor receptor-bound protein 2; IP: immunoprecipitation; IRS-1: insulin receptor substrate 1; MAPK: mitogen-activated protein kinase; PI3-K: phosphatidylinositol 3´-kinase; SH: Src homology

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Correspondence
Celestino González González
Departamento de Biología Funcional (Fisiología)
Universidad de Oviedo
C/Julián Clavería s/n
3006 Oviedo
Spain
Phone: +34-985-104.209
Fax: +34-985-103.534
E-mail address: tinog@correo.uniovi.es

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