Dose- and time-dependent effects of 17 β -oestradiol on insulin sensitivity in insulin-dependent tissues of rat: implications of IRS-1

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Abstract

Numerous studies have suggested that ovarian hormones are able to modulate insulin sensitivity, but their exact role remains unclear. We have investigated whether different doses of 17 β -oestradiol mediate changes in insulin sensitivity and if these changes could be related to modifications of insulin receptor substrate-1 (IRS-1). Female rats were ovariectomized and later separated into three groups: untreated; treated with a dose of 17 β -oestradiol sufficient to reproduce gestational plasma concentrations of 17 β -oestradiol (group E); and treated with a dose 100 times greater than that given to group E (group E2). A euglycaemic-hyperinsulinaemic clamp was used to measure insulin sensitivity. Changes in IRS-1 were analysed by Western blotting and RT-PCR assays. In group E we found a decrease in insulin sensitivity between

days 11 and 16 of treatment as in late gestation, whereas in the untreated group and group E2, development of insulin resistance was observed throughout the treatment. In contrast, whereas in group E2 insulin resistance throughout the hormonal treatment was related to diminished expression and phosphorylation of IRS-1, in group E the decrease in insulin sensitivity between days 11 and 16 of treatment was not related to a decrease in IRS-1 expression. Our results suggest that the effects of oestradiol on insulin sensitivity were dose-dependent and that the insulin resistance associated with a high dose of 17 β -oestradiol was related to downregulation of IRS-1 expression.

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Introduction

Insulin resistance can be defined as a complex nutritional–metabolic state characterized by lower sensitivity of target tissues (liver, skeletal muscle and adipose tissue) to the physiological effects of insulin (Kaaks & Lukanova 2001). Thus insulin resistance is considered as a common aetiological factor for the individual components of the metabolic syndrome, comprising hypertension, dyslipidaemia with or without hyperglycaemia, and abnormalities in the homeostatic system (Writing Group for the PEPI Trial 1995, Fagan & Deedwania 1998). Individuals with this syndrome are at high risk for cardiovascular disease and type II (non-insulin-dependent) diabetes mellitus (NIDDM) (DeFronzo & Ferrannini 1991, Reaven 1993).

Various clinical observations and experimental data suggest that insulin and sex hormones interact (Kumagai *et al.* 1993, Polderman *et al.* 1994). Thus the low levels of female sex steroids associated with the menopause (natural or surgical) are related to a decrease in whole-body insulin-mediated glucose uptake. The cellular mechanism behind this insulin resistance and the role of low levels of female sex steroids are not yet fully understood (Rincón

et al. 1996). Many studies have found that oestrogen replacement therapy seems to improve insulin sensitivity in postmenopausal women (Godsland 1996, Colacurci *et al.* 1998, Karjalainen *et al.* 2001). However, the effects of oestrogen replacement therapy seem to be different depending on the dose, route and type of oestrogen utilized. In this respect, high doses of oral oestrogens (1.25 mg/day) have been reported to cause a deterioration in, and low doses (0.625 mg/day) to exert no or even beneficial effects on insulin–glucose metabolism (Cagnacci *et al.* 1992, Lindheim *et al.* 1993). However, both a low dose (0.05 mg/day) and a high dose (1 mg/day) of transdermal oestradiol improved insulin sensitivity (Lindheim *et al.* 1994), suggesting that the adverse effect of a high oral dose could be explained by first-pass metabolism in the liver (Krauss *et al.* 1988, Cagnacci *et al.* 1992). In women with NIDDM, the changes that accompany menopause may further diminish glycaemic control and insulin sensitivity (Ferrara *et al.* 2001). The beneficial effects on insulin sensitivity conferred by low-dose oestradiol replacement therapy in healthy postmenopausal women could be a source of particular benefit to diabetic postmenopausal women, some authors (Andersson *et al.*

1997, Brussard *et al.* 1997) suggesting that low doses of oestradiol improve glycaemic control in these women. Furthermore, the predominant mechanism for these improvements seems to be an enhancing effect of oestradiol on insulin sensitivity in the liver (Sattar 1998).

An early event in insulin action, after receptor binding, is the activation of tyrosine kinase activity in the cytosolic domain of the receptor (Goldfine 1987). Insulin receptor substrate-1 (IRS-1) is a major endogenous substrate of the insulin receptor kinase and undergoes tyrosine phosphorylation upon insulin stimulation, after which it serves as a docking protein for a number of Src homology 2 domain-containing proteins such as Grb2, Syp, Nck and the two isoforms of the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (White & Kahn 1994, Virkamaki *et al.* 1999). Although IRS-1 appears to have no catalytic function, it may act as a docking protein, bringing various component proteins of the insulin signalling pathway into proximity and forming a multifactorial signalling complex (Sun *et al.* 1992, Water & Pessin 1996). These interactions are essential tools for the transmission and compartmentalization of the insulin signal from the receptor to the final cellular events such as stimulating glucose uptake, activating glycogen or protein synthesis, and initiating specific gene transcription (Virkamaki *et al.* 1999).

The classical mechanism of oestradiol action involves an intracellular receptor, which upon hormone binding assumes an activated state. This hormone-receptor complex is a transcriptionally active protein that is able to modulate gene expression (Beato 1989). However, the molecular mechanism responsible for the relationship between oestradiol and insulin sensitivity remains unclear. In most cases, investigations carried out *in vitro* have shown that oestradiol can increase the expression and phosphorylation of IRS-1 in human breast cancer cells (Lee *et al.* 1999, Molloy *et al.* 2000) or that oestradiol can decrease insulin-stimulated glucose transport by diminishing the concentrations of IRS-1 (Collison *et al.* 2001) in 3T3-L1 adipocytes. However, in some *in vivo* experiments, glucose metabolism in peripheral tissues was believed to be regulated in manner different from that in the liver (Dunaif *et al.* 1992, Huang *et al.* 1996). We consider it very difficult to reconcile these findings with whole-body metabolism. To our knowledge, there are no reports on the role of oestradiol in the modulation of the insulin signalling cascade on the principal insulin-sensitive tissues (liver, skeletal muscle and adipose tissue). More specifically, the possible direct action of oestradiol on the IRS-1 gene and protein regulation in *in vivo* studies is unknown.

In this work, we have investigated whether two different doses of 17 β -oestradiol mediate changes in insulin sensitivity in ovariectomized rats, and whether these changes could be related to modifications in the amount of IRS-1, phosphorylation of this protein, or expression of

the IRS-1 gene in the principal insulin-sensitive tissues: liver, skeletal muscle and adipose tissue.

Materials and Methods

Animals

We studied 12-week-old virgin female Wistar rats (from the University of Oviedo animal house) weighing 250–280 g and kept under standard conditions of temperature ($23 \pm 3^\circ\text{C}$) and humidity ($65 \pm 1\%$), and a regular lighting schedule of 12 h light:12 h darkness (lights on 0800–2000 h). The animals were fed a standard diet (Panlab A04) and had free access to water. All experimental manipulations were performed between 0930 h and 1230 h. The experiments were carried out in accordance with principles of laboratory animal care.

Experimental design

Three days before the hormonal treatment was initiated (day –7), the rats were ovariectomized through a midline incision under light ether anaesthesia. Ovariectomized rats were separated randomly into three groups: untreated group (V), group E and group E2, and housed individually throughout the experiment.

After surgery, ovariectomized rats 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 (0900 h and 2100 h) for 20 days, with 0.1 ml of a suspension of 17 β -oestradiol (Sigma Chemical Co., St Louis, Missouri, USA) in olive oil/ethanol (3:2 v/v). The control (untreated) group were injected with vehicle (olive oil/ethanol 3:2 v/v) in parallel. In group E, different doses of 17 β -oestradiol were injected (González *et al.* 1998, 2002b) in order to simulate the plasma concentrations that we observed in normal pregnant rats (González *et al.* 1997). The doses of 17 β -oestradiol injected in group E2 were 100 times those injected in group E.

The hormonal treatment was applied according to the schedule shown in Fig. 1.

The three groups were divided randomly into three subgroups of six animals each, named group 6, group 11 and group 16 according to the day of the experimental procedure on which the animals were killed. These days were selected because changes have been found in sensitivity to the action of insulin during pregnancy in the Wistar rat (González *et al.* 1997).

Euglycaemic insulin clamp

Clamp experiments were performed in anaesthetized rats by a procedure described previously (González *et al.* 2000). Before the clamp began, and in order to determine basal

Ovariectomy

DAYS	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6*
[E]	-	-	-	EA	EA	EA	EA	EA	EA	EA	EA	EA	EA	EB
[E2]	-	-	-	E2A	E2A	E2A	E2A	E2A	E2A	E2A	E2A	E2A	E2A	E2B

DAYS	7	8	9	10	11*	12	13	14	15	16*
[E]	EB	EB	EB	EB	EA	EA	EA	EA	EA	
[E2]	E2B	E2B	E2B	E2B	E2A	E2A	E2A	E2A	E2A	

EA=0.006 mM; EB=0.003 mM; E2A=0.6 mM; E2B=0.3 mM

Figure 1 Schedule for hormonal treatment with 17 β -oestradiol. *Day on which animals were killed for study. [E], oestradiol concentration.

insulin concentration, a blood sample (1 ml) was collected from the jugular vein into heparinized tubes and centrifuged at 3000 r.p.m. for 20 min at 4 °C, then plasma was immediately drawn off and stored frozen at -20 °C until required for assay. 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).

After the clamp study, blood samples (4 ml) for the determination of final insulin and 17 β -oestradiol plasma concentrations were collected from the jugular vein into heparinized tubes, centrifuged at 3000 r.p.m. for 20 min at 4 °C and plasma immediately drawn off and stored frozen at -20 °C until required for assay. The total volume of blood taken from each animal was 5.5–6.5 ml. Plasma insulin was measured by RIA using a kit (DGR Instruments GmbH, Marburg, Germany) for rat insulin. The sensitivity of the assay was 0.1 ng/ml, and the intra-assay coefficient of variation was 9.32%. The sample was assayed in duplicate. Plasma 17 β -oestradiol was measured by RIA using Immuchen kits (ICN Biomedicals Inc., Orangeburg, NY, 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. Finally, samples of different tissues were collected and immediately frozen in liquid nitrogen for future experiments and animals were killed by bleeding.

Analysis of RNA by semiquantitative RT-PCR

The RT-PCR, optimized for semiquantitative detection, was used to analyse overall levels of expression of IRS-1 mRNA in each individual animal of each different experimental group. Total RNA from the liver, skeletal muscle (flexor digitorum superficialis, extensor digitorum longus, soleus and extensor digitorum lateralis) and retroperito-

neal adipose tissue samples of each individual animal were isolated by the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987) and treated with RNase-free DNase I (Amersham Pharmacia Biotech) in order to remove genomic DNA. For amplification of the targets we used the following oligonucleotides deduced from cloned rat IRS-1 (Gosbell *et al.* 2000): 5'-CCG GAA TTC GCC AAT CTT CAT CCA GTT GCT and 5'-CCC AAG CCT CAT CGT GAA GAA GGC ATA GGG. It is noteworthy that this primer pair was designed to amplify a 350 bp fragment of rat IRS-1 cDNA. In addition, in order to provide an appropriate internal control, coamplification of a 540 bp fragment of the β -actin mRNA was carried out in each sample using the following oligonucleotides deduced from cloned rat actin: 5'-CCG GAA TTC CGT GGG CCG CCC TAG GCA and 5'-CGC GGA TCC TTT AAT GTC AGG GAC GAT.

For amplification of the target, RT and PCR were run in two separate steps. Furthermore, to enable appropriate amplification in the exponential phase for each target, PCR amplifications of IRS-1 and β -actin were carried out in separate reactions with a different number of cycles but using similar amounts of the corresponding cDNA templates, generated in single RT reactions. Briefly, equal amounts of total RNA (5 μ g) were heat-denatured and reverse-transcribed by incubation at 42 °C for 45 min using an RNA PCR Core Kit (Perkin Elmer Corp., Branchburg, NJ, USA) in a final volume of 30 μ l. The reactions were terminated by heating at 99 °C for 5 min and finally at 4 °C for 5 min. For semiquantitative PCR, 10 μ l aliquots of the cDNA samples (equivalent to 650 ng total RNA input) were amplified using an RNA PCR Core Kit (Perkin Elmer Corp.) and the appropriate primer pairs. The number of cycles was optimized to ensure amplification in the exponential phase of PCR. Finally, we used 30 cycles of PCR amplifications to detect the IRS-1

mRNA in adipose tissue and 28 cycles for liver and skeletal muscle. Conditions for the amplification were 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C. Also, we used 30 cycles of PCR amplifications to detect β -actin mRNA in all tissues. Conditions for the amplification were 15 s at 94 °C, 15 s at 55 °C and 45 s at 72 °C.

The cDNA fragments generated were resolved in 0.8% agarose gel containing ethidium bromide (0.1 μ g/ml) and visualized and photographed with u.v. transillumination, their molecular sizes being determined by comparison

with size markers run together with the cDNA products. Specificity of PCR products was confirmed by digestion with specific restriction endonuclease (Taq I, MBI Fermentas Inc., Amherst, NY, USA). For quantitative evaluation, absolute optical densities of RT-PCR signals were obtained by densitometric scanning using an image analysis system (NIH Image 1.57 software). IRS-1 mRNA is expressed as a ratio: mRNA IRS-1: β -actin mRNA. Moreover, in order to ensure that equal inputs of RNA were added to RT-PCR reactions, only samples yielding roughly similar absolute optical density values for β -actin bands were considered for further analysis. In addition, to minimize potential RT-PCR artefacts due to inherent reaction variability, all data points were repeated at least three times for each sample, using independent RNA samples. Finally, in all assays, liquid controls and reactions without RT were included, yielding negative amplifications.

Immunoprecipitation and Western blotting

The samples of liver, skeletal muscle and adipose tissue of each animal were washed with ice-cold PBS and homogenized immediately in 3 ml lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40 Roche Diagnostics, 0.05% sodium deoxycholate, 0.1% orthovanadate 1 M) at 4 °C using a Polytron (PT-MR-3000, Kinematic AG, Lucerne, Switzerland) operated at maximum speed for 30 s. The extracts from each individual animal were centrifuged at 12 000 *g* at 4 °C for 10 min in order to remove insoluble material. After centrifugation, the protein content was assayed by the Bradford dye-binding method (Bradford 1976) using BioRad 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. The aqueous fraction containing 250 μ g protein for liver and muscle and 150 μ g for adipose tissue was used for immunoprecipitation with 0.25 μ g polyclonal

Figure 2 (a) Plasma concentrations of 17 β -oestradiol in ovariectomized rats (V) and rats treated with different doses of 17 β -oestradiol (E, E2). (b) Comparison of increase in body weight in a control (vehicle-treated) group (V) and in rats treated with different doses of oestradiol (E, E2). A, B, C: changes in body weight from day 0 to 6, day 6 to 11 and day 11 to 16 respectively. (c) Comparison of glucose infusion rates in ovariectomized rats treated with vehicle (V) or with different doses of oestradiol (E, E2). The rate of infusion of glucose was assessed as the mean values from 40 to 60 min during euglycaemic-hyperinsulinaemic clamp experiments. Values are means $\pm$ S.E.M. for six animals. Solid bar, vehicle-treated group (V); open bar, group E; striped bar, group E2. Significant differences between treatment groups ($P \leq 0.05$) are listed for each experimental time point (intraday comparisons). Significant differences within treatment groups: *16 vs 6,11; $\heartsuit$ 6 vs 11,16; $\spadesuit$ 11 vs 16; *A vs C.

Table 1 Fasting blood glucose, fasting serum insulin and serum insulin after clamp experiment in rats treated with vehicle (V) or with different doses of oestradiol (E, E2) on days 6, 11 and 16 of the experiment

	Day 6	Day 11	Day 16	Significant differences
Fasting blood glucose (mg/dl)				
V	108.77 $\pm$ 4.01	110.00 $\pm$ 3.00	113.16 $\pm$ 2.38	
E	118.50 $\pm$ 5.31	108.33 $\pm$ 4.69	117.51 $\pm$ 4.44	
E2	122.00 $\pm$ 5.68	120.12 $\pm$ 5.39	116.71 $\pm$ 6.79	
Fasting serum insulin (ng/ml)				
V	0.67 $\pm$ 0.02	0.73 $\pm$ 0.02	0.85 $\pm$ 0.03	16 vs 6, 11
E	1.03 $\pm$ 0.03	0.52 $\pm$ 0.01	0.74 $\pm$ 0.02	16 vs 6, 11; 6 vs 11
E2	1.60 $\pm$ 0.05	1.01 $\pm$ 0.03	1.33 $\pm$ 0.024	16 vs 6, 11; 6 vs 11
Comparison	E2 vs V, E E vs V	E2 vs V, E E vs V	E2 vs V, E E vs V	
Serum insulin post clamp[†] (ng/ml)				
V	2.15 $\pm$ 0.55	7.45 $\pm$ 1.97	11.54 $\pm$ 3.78	16 vs 6, 11; 11 vs 6
E	5.18 $\pm$ 1.25	3.85 $\pm$ 0.78	1.99 $\pm$ 0.37	6 vs 11, 16; 11 vs 16
E2	2.84 $\pm$ 0.55	1.54 $\pm$ 0.32	2.41 $\pm$ 0.73	6 vs 11, 16; 11 vs 16
Comparison	E2 vs V, E E vs V	V vs E, E2 E vs E2	V vs E, E2	

Values are means $\pm$ S.E.M. for six animals per group. [†]After clamp experiments. Only significant differences are shown.

antibody against IRS-1 (sc-559; Santa Cruz Biotech., Inc. Santa Cruz, CA, USA). The immune-complexes were precipitated with protein G-agarose beads (Roche Diagnostics) overnight at 4 °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% orthovanadate 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 -mercaptoethanol, bromophenol blue) and heated in a boiling water bath for 5 min. Efficacy of each immunoprecipitation was 100%, as confirmed by Western blot analysis of a second round of immunoprecipitation, which yielded no detectable IRS-1 protein.

For total extract, duplicate similar-sized aliquots were subjected to SDS-PAGE (7% Tris-Acri-Bis) in a BioRad miniature slab gel apparatus. The prestained molecular mass standards used were myosin (218 kDa), b-galactosidase (126 kDa), BSA (90 kDa), carbonic anhydrase (43.5 kDa), soybean trypsin inhibitor (33.9 kDa), lysozyme (17.4 kDa) and aprotinin (7.6 kDa) (BioRad). Electrophoretic transfer of proteins from the gel to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia Biotech) was performed for 60 min at 50 V (constant) in a BioRad miniature transfer apparatus (Mini-Protean) as described by Towbin *et al.* (1979). Adult rat skeletal muscle protein was used as a positive control for IRS-1 protein.

Non-specific binding of protein to the nitrocellulose membranes was reduced by preincubating the filter for 2 h at room temperature in blocking buffer (TNT, 7%

BSA), and probing with a 1:75 000 dilution of anti-phosphotyrosine antibody (sc-7020, Santa Cruz Biotech., Inc.) conjugated to horseradish peroxidase for 1 h at room temperature, diluted in blocking buffer. The membranes were rinsed several times with blocking buffer without BSA and proteins were detected with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions and using autoluminography on Kodak X-Omat film.

Later, the membranes were incubated for 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. After this, another Western blot analysis was performed (Western-Light, Chemiluminescent Detection System, Tropix, Inc., Bedford, MA, USA) using a 1:5000 dilution of polyclonal antibody against IRS-1 as the primary antibody, followed by alkaline-phosphatase-conjugated anti-rabbit IgG antibody (Tropix, Inc.) for detection. Finally, the proteins were detected by autoluminography on Kodak X-Omat film.

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

Statistics

Data are expressed as means $\pm$ S.E.M. Within-group comparisons for the period of hormonal treatment were made using analysis of variance (ANOVA) or Kruskal-Wallis one-way ANOVA, and the Student-Newman-Keuls test or Mann-Whitney U-Wilcoxon rank sum W test. A value of $P \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS for Windows version 6.01.

Figure 3 Representative experiments for expression analysis of IRS-1 in (a) liver, (b) skeletal muscle, and (c) adipose tissue in control rats (solid bars, V) and oestradiol-treated rats (open bars, group E; shaded bars, group E2) at days 6, 11 and 16 of treatment. Left: RT-PCR was performed on 5 μ g RNA from the samples. A 350 bp fragment corresponding to a segment of IRS-1 was amplified in a volume of 100 μ l, and 10 μ l of the reaction was separated on a 0.8% agarose gel. A 540 bp fragment corresponding to a β -actin was amplified as an internal control. Reactions without the RNA sample were performed as negative controls (lane NC). Right: Relative abundance of IRS-1 mRNA. The results were corrected by the endogenous levels of β -actin mRNA. Data are expressed as means $\pm$ S.E.M. A.S.U., arbitrary scanning units. Significant differences between treatment groups are listed for each experimental time point (intraday comparison). Within the treatment groups, the following significant differences ($P < 0.05$) were identified over time: ♥ 6 vs 11, 16; ♦ 11 vs 16; ♣ 11 vs 6, 16; ♠ 16 vs 6, 11.

Results

Plasma concentrations of 17 β -oestradiol are shown in Fig. 2a. The concentrations in group E were similar to those in normal pregnant rats at 5, 10 and 15 days of pregnancy (González *et al.* 1997). In this group, we found similar values at 6 and 11 days of treatment and a significant increase between 6–16 and 11–16 days of treatment. The concentrations in group E2 were significantly greater than those in group E and the untreated group. The plasma concentrations of 17 β -oestradiol were dependent on the solution injected, thus we observed that the plasma concentration of 17 β -oestradiol increased as follows: at day 6, 238% in group E compared with the untreated group, and 787% in group E2 compared with the untreated group; at day 11, 188% in group E compared with the untreated group, and 1145% in group E2 compared with the untreated group; at day 16, 401% in group E compared with the untreated group, and 1463% in group E2 compared with the untreated group.

Comparisons of increase in body weight can be seen in Fig. 2b. The increase in body weight was significantly greater on days 0–6 of the experimental period in the untreated group compared with days 11–16. No differences were noted in the other groups. On days 0–6 (Fig. 2b, A), the increase in body weight was significantly greater in the untreated group than in the other groups. On days 6–11 (Fig. 2b, B) the increase in body weight was significantly greater in the untreated group than in group E2. On days 11–16 (Fig. 2b, C) the increase in body weight was significantly greater in the untreated group than in groups E and E2.

To investigate insulin resistance in the rats at different days of the hormonal treatment, glucose clamp experiments were carried out under euglycaemic and hyperinsulinaemic conditions. Figure 2c shows the comparison of glucose infusion rates in different groups of rats treated with different doses of 17 β -oestradiol. At days 6, 11 and 16, the insulin sensitivity was significantly greater in group E than in the other groups. Studying the effect of the hormonal treatment, we were able to observe that in the untreated group the insulin sensitivity did not change throughout treatment. In group E2 the sensitivity to the action of insulin decreased significantly in a gradual fashion between days 6 and 16; in group E we found that the insulin sensitivity remained without significant change during the first 11 days of treatment and decreased significantly between days 11 and 16. In addition, we must point out that insulin sensitivity was always greater in the untreated group than in group E2, but these differences are significant only at day 11 of the experiment. These results demonstrate that group E was significantly more sensitive to the action of insulin than the other groups, and that this group was less sensitive at the end of hormonal treatment (day 16) than in the early period of hormonal treatment (days 6 and 11).

Table 1 shows the values of fasting blood glucose and fasting insulin plasma concentrations before and after clamp experiments. Fasting blood glucose concentrations were observed to be similar between groups and between days of the experimental period.

In the untreated group, the serum concentrations of insulin before the clamp study increased significantly throughout the experiment, whereas groups E and E2 had a similar insulin concentration profile, and in both groups this parameter decreased significantly between days 6 and 11 and increased significantly between days 11 and 16. In contrast, group E2 had significantly greater insulin concentrations than the others on any day of treatment.

After clamp experiments, the insulin plasma concentrations increased significantly throughout the experiment in the untreated group, whereas in group E this parameter decreased significantly throughout the treatment. In group E2, the plasma concentrations of insulin after the clamp decreased between days 6 and 11 and increased between days 11 and 16. At day 6 of treatment, the insulin concentrations after the clamp were significantly greater in group E than in the other groups, whereas at days 11 and 16 this parameter was significantly greater in the untreated group than in the other groups.

An initial Northern blot analysis of IRS-1 mRNA revealed, in agreement with the findings of Araki *et al.* (1995), a low abundance of IRS-1 mRNA in the tissues studied, so that quantification was not possible. Therefore, semiquantitative RT-PCR was performed to determine the level of expression of the *IRS-1* gene (Fig. 3).

The hepatic IRS-1 mRNA levels (Fig. 3a) were significantly lower in group E2 than in the other groups at days 11 and 16 of treatment. The changes in this parameter through the experimental period were similar in the oestradiol-treated groups between days 11 and 16, when mRNA for IRS-1 decreased significantly. However, there were significant increases between days 6 and 11 in group E, but no changes in group E2. In the untreated group, the IRS-1 mRNA increased significantly throughout the experimental period.

In skeletal muscle (Fig. 3b), changes in the IRS-1 mRNA levels were not found at either the intra- or intergroup level.

In adipose tissue (Fig. 3c), the mRNA levels of IRS-1 were significantly greater in group E than in the other groups at day 6 of treatment, whereas at days 11 and 16 group E2 exhibited lower IRS-1 mRNA levels than the other groups. In contrast, in group E there were no significant changes throughout treatment, whereas in group E2 these mRNA levels showed significant decreases between days 11 and 16. In the untreated group, the levels of IRS-1 mRNA increased significantly throughout the experimental treatment.

Figure 4 shows a representative experiment in which solubilized liver, skeletal muscle and adipose tissue material were subjected to immunoprecipitation using

an anti-IRS-1 antibody. After SDS-PAGE and electro-transfer, nitrocellulose membranes were incubated in the presence of anti-phosphotyrosine antibody. ECL detection showed, in the materials immunoprecipitated, one band of 185 kDa corresponding to the IRS-1. Later, the membranes were stripped and incubated in the presence of anti-IRS-1 antibody.

In liver, group E showed significantly greater IRS-1 levels than group E2 and the untreated group at any day of treatment (Fig. 4aI). Over the period of treatment, hepatic IRS-1 levels increased significantly in group E between days 6 and 11 and decreased between days 11 and 16. In group E2, the amount of IRS-1 decreased significantly throughout the period of hormonal treatment, whereas in the untreated group the IRS-1 levels increased significantly with the duration of treatment. The phosphorylation of IRS-1 in liver (Fig. 4aII) had a pattern similar to that of the amount of IRS-1.

At day 6 of treatment in skeletal muscle (Fig. 4bI), group E showed significantly greater concentrations of IRS-1 than the other groups; at day 11 no differences were found in these concentrations, and at day 16 group E2 showed a significantly greater amount of IRS-1 than the untreated group and group E. In group E, the duration of treatment was associated with a significant decrease in IRS-1 concentrations between days 6 and 11, whereas between days 11 and 16 these concentrations increased significantly. The amount of IRS-1 in group E2 increased significantly throughout the hormonal treatment, and in the untreated group the IRS-1 concentrations increased significantly between days 6 and 11. In contrast, the phosphorylation of IRS-1 in skeletal muscle (Fig. 4bII) showed a similar profile in group E2, increased significantly between days 6 and 11 and decreased between days 11 and 16 in the untreated group, and exhibited no significant changes in group E.

The amount of IRS-1 in adipose tissue (Fig. 4cI) was significantly greater in group E than in the other groups at day 6 of treatment, whereas at days 11 and 16 this amount was significantly lower in group E2 than in the other groups. Over the period of hormonal treatment, group E exhibited a significant decrease in IRS-1 concentrations between days 6 and 11, whereas these concentrations increased significantly between days 11 and 16. In group E2, the duration of treatment was associated with a significant decrease in IRS-1 concentrations, whereas in the untreated group there was a significant increase throughout the experiment. In contrast, phosphorylation of IRS-1 in

adipose tissue (Fig. 4cII) increased significantly throughout the treatment period in the untreated group, but decreased in group E2. In group E, IRS-1 phosphorylation increased significantly between days 11 and 16.

Discussion

Several lines of evidence have revealed an association between female sex steroids and insulin sensitivity (Kumagai *et al.* 1993, Polderman *et al.* 1994). In addition, several clinical reports have shown that artificially or naturally increased oestradiol concentrations are linked to changes in insulin sensitivity (Cagnacci *et al.* 1992, Godsland 1996, Rincón *et al.* 1996, Colacurci *et al.* 1998, Karjalainen *et al.* 2001). In this study, we have investigated whether oestradiol had any effects on insulin sensitivity in ovariectomized rats treated with this steroid. We chose two different doses of 17 β -oestradiol – a physiological dose (group E) that promotes gestational oestradiol concentrations (González *et al.* 2000) and a pharmacological dose (group E2) – and examined insulin sensitivity using a hyperinsulinaemic–euglycaemic clamp method (González *et al.* 2000). In group E, we were able to see a decrease in insulin sensitivity at the end of treatment (between days 11 and 16) (Fig. 2c), which reflects the events of late gestation. In this way, we and others (Herrera *et al.* 1991, González *et al.* 1998, 2002a) have shown that there is a decrease in insulin sensitivity at late gestation, and this insulin resistance at the end of gestation determines a competitive advantage for the fetuses because uterine glucose uptake and placental glucose transport are relatively unaffected by maternal insulin (Rankin *et al.* 1986). Present results on the clamp in group E (Fig. 2c) suggest that the gestational dose of 17 β -oestradiol seems to be able to reproduce the changes in insulin sensitivity that are induced by pregnancy (González *et al.* 1998, 2002b).

However, the results in group E2 show that pharmacological doses of 17 β -oestradiol promote the development of insulin resistance from the early treatment period (Fig. 2c). These findings are in agreement with those of most of the authors who have reported that high doses of oestradiol are associated with insulin resistance (Barret-Connor & Laakso 1990, Troisi *et al.* 2000). Moreover, according to our findings in the oestradiol-treated groups (E and E2), the effects of this steroid on insulin sensitivity seem to be dose-dependent. If we analyse the changes in insulin sensitivity in oestradiol-treated groups, we can see that

Figure 4 Representative experiments for measurement of IRS-1 protein concentrations and phosphorylation in (a) liver, (b) skeletal muscle and (c) adipose tissue in control rats (solid bars, V) and oestradiol-treated rats (open bars, group E; shaded bars, group E2). The proteins were isolated with lysis buffer, as described in Methods; after centrifugation, aliquots of the supernatant were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-phosphotyrosine antibody. Later, the membranes were incubated with stripping buffer and immunoblotted with anti-IRS-1 antibody. Data are expressed as means $\pm$ S.E.M. A.S.U., arbitrary scanning units. Significant differences between treatment groups are listed for each experimental time point (intraday comparison). Within the treatment groups, the following significant differences ($P < 0.05$) were identified over time: *6 vs 11, 16; *11 vs 16; *11 vs 6, 16; *16 vs 6, 11.

there is a difference in insulin sensitivity between group E (lower dose) and group E2 (higher dose), in line with the findings in another study (Linheim *et al.* 1993) and confirming our above hypothesis. However, in the ovariectomized control group, there was a progressive impairment in insulin sensitivity throughout treatment. These observations are in agreement with those of others (Kumagai *et al.* 1993, González *et al.* 2000) who have shown that the absence of female sex steroids results in a decreased insulin sensitivity.

We also investigated whether the molecular mechanism underlying these changes in insulin sensitivity is related to IRS-1. In the past few years, considerable advances in knowledge have been made concerning the insulin intracellular signalling pathways, and IRS-1 represents the epicentre for the majority of insulin receptor downstream signalling events involved in the actions of insulin (White 1998). In group E, we found a decrease in insulin sensitivity between days 11 and 16 similar to that in late gestation (Fig. 2c). However, when we analysed the changes in IRS-1 expression we did not find a straightforward relationship between this parameter and the changes in insulin sensitivity (Fig. 3): when the insulin sensitivity decreased between days 11 and 16 of treatment, the amount of IRS-1 and its phosphorylation significantly increased in peripheral tissues (skeletal muscle and adipose tissue) (Fig. 4b and c) and decreased in the liver (Fig. 4a). These results do not seem to be in line with those of a previous study in which we showed that insulin receptor performance in rats treated with a gestational dose of 17 β -oestradiol was related to changes in insulin sensitivity (González *et al.* 2002b) in a tissue-specific manner. In this context, we think that the regulation of IRS-1 by 17 β -oestradiol could be mediated in a manner different from that of the insulin receptor, because IRS-1 has a pivotal role in several pathways such as leptin (Szanto & Kahn 2000), growth hormone (GH) (Liang *et al.* 2000) and prolactin (Berlanger *et al.* 1997). Although the biological endpoints of these endocrine pathways are quite different, there are several overlapping points during the intermediate steps comprising the signal transduction machinery, which involves considerable intracellular compartmentalization. In the past few years several reports (Clark *et al.* 1998, Whitehead *et al.* 2000) have shown that IRS-1 could be localized in two different pools: the cytoskeletal component and the cytosol. In this respect, the mode of IRS-1 recruitment is quite different between insulin and other hormones: insulin mediates IRS-1 phosphorylation through the tyrosine kinase activity of a membrane insulin receptor (Sun *et al.* 1992, White & Kahn 1994, Virkamaki *et al.* 1999), whereas the other hormones use a family of cytoplasmic tyrosine kinases termed Janus kinases (Berlanger *et al.* 1997, Liang *et al.* 2000, Szanto & Kahn 2000).

It has been argued that the cytoskeletal component represents the functional pool of IRS-1 for the insulin

signalling pathway, because it provides a platform for locating IRS-1 proteins within the proximity of the membrane insulin receptor. Olson *et al.* (2001) recently proposed that the insulin signalling cascade leading specifically to glucose transporter 4 vesicle recruitment is located in microtubules of the cytoskeleton and, in agreement with these authors, it has been reported (Clark *et al.* 1998) that there is a fraction of PI3K that is also located on the cytoskeleton. The microtubule network, which forms the cytoskeletal scaffold, may ensure the correct juxtaposition of different enzymes in the insulin signalling cascade, enabling them to interact in a very robust manner. Moreover, it has been reported that the inappropriate release of the IRS-1 proteins to the cytosol causes insulin resistance (Clark *et al.* 2000). Thus it has been shown that treatment with oestradiol determines a decrease in insulin sensitivity through a mechanism that involves a release of IRS-1 proteins from the intracellular cytoskeleton (Collison *et al.* 2001). With the clamp experiments, insulin resistance means that there is a low glucose uptake in response to insulin stimulation and that IRS-1 has effects on glucose transport only when it is recruited by the insulin receptor. Taking these data together with our own findings, we postulated that the decrease in insulin sensitivity in group E between days 11 and 16 of treatment could be related to a release of IRS-1 protein from the cytoskeletal scaffold, where it is able to interact with the insulin receptor and pass to the cytosol, where it could be recruited by another hormonal signalling pathway. However, our results on IRS-1 seem not to confirm those of Saad *et al.* (1997), who reported a decrease in the expression of IRS-1 in skeletal muscle during late pregnancy. We consider that these differences could have various explanations. In our experimental design, we ensured that no other gestational hormones were present that could regulate the expression of IRS-1, because this protein could be implicated in its own intracellular signalling pathway. Moreover, the experiments by Saad's group were relevant to the end of gestation (day 20–21), whereas in our oestradiol treatment we simulated gestational plasma concentrations of oestradiol up to day 16 of gestation (Fig. 2a).

In the clamp experiments, the E2 and the untreated groups were similar in that insulin sensitivity decreased throughout the experimental period (Fig. 2c). However, analysis of the results for IRS-1 revealed a different pattern: in liver and adipose tissue, IRS-1 mRNA, protein and phosphorylation decreased significantly in group E2 throughout the experiment, whereas in the untreated group they increased (Figs 3 and 4). We think that insulin resistance in the untreated group and that in group E2 have different molecular mechanisms. In group E2, the development of insulin resistance throughout the experimental period could be related to a decrease in the expression of IRS-1 in liver and adipose tissue, in agreement with the findings of other authors (Carvalho *et al.*

1996, 1999). This is in agreement with previous work in which we demonstrated that a pharmacological dose of 17 β -oestradiol mediates a significant decrease in insulin receptor (González *et al.* 2002b). It seems that pharmacological doses of 17 β -oestradiol determine insulin resistance through decreases in the first steps of the insulin signalling system. We believe that the effect on the expression of insulin receptor and the *IRS-1* gene could be due to the possible existence of the consensus response elements to oestradiol in the insulin receptor and *IRS-1* gene promoter. Probably, the high plasma concentration of oestradiol in group E2 was determining the binding of many complexes of oestradiol receptor–oestradiol to the insulin receptor and *IRS-1* promoter, and this fact could determine the linking of these complexes or the displacement of the other transcription factors such as lactogenic hormones, GH, etc. We are currently testing this hypothesis. It is also possible that a pharmacological dose of 17 β -oestradiol could be implicated in a release of *IRS-1* proteins from the cytoskeleton to the cytosol, which would increase the development of insulin resistance.

In contrast, in the untreated group there was no relationship between the development of insulin resistance and changes in *IRS-1* and insulin receptor expression (González *et al.* 2002b). Present findings in the untreated group are in agreement with the hypothesis of oestradiol receptor–oestradiol complexes mentioned above. Oestradiol seems to be able to mediate changes in insulin sensitivity, and so the absence of oestradiol in the untreated group determined the absence of the oestradiol receptor–oestradiol complexes, and regulation of insulin receptor and *IRS-1* gene expression was not related to changes in insulin sensitivity in the untreated group. However, we cannot exclude the possibility that the absence of female sex steroids could be implicated in changes in the intracellular distribution of *IRS-1*, and this could be related to a decrease in insulin sensitivity. In this respect, the results for body weight also show different metabolic performance in the untreated group and group E2, the weight gain in the untreated group being significantly greater than that in group E2 (Fig. 2b). Many studies have demonstrated that the absence of female sex hormones determines an increase in body weight in rats (Kumagai *et al.* 1993, González *et al.* 2000), monkeys (Wagner *et al.* 1998) and humans (Tremollieres *et al.* 1996), and this increase could be prevented by oestradiol treatment (Fineberg 2000, González *et al.* 2000). However, this effect of 17 β -oestradiol seems to be independent of the dose, because we did not find differences in body weight gain between groups E and E2. We did not measure plasma leptin concentrations, but the mechanism responsible for these changes in body weight is likely to be related to this hormone. Leptin is the main regulator of feeding behaviour (Szanto & Kahn 2000). It has been reported that loss of ovarian function determines an increase in food intake (Wade 1975), and that

ovariectomy-induced weight gain is caused by a decrease in leptin concentration (Chu *et al.* 1999), which could be prevented by 17 β -oestradiol treatment (Kristensen *et al.* 1999). In this context, it has been proposed that leptin and insulin signalling networks may be connected at the level of *IRS-1* (Szanto & Kahn 2000), but most evidence seems to suggest that there is a tissue-specific interaction between leptin and insulin that is quite variable. However, the lipolytic effects of 17 β -oestradiol in controlling adipose lipoprotein lipase cannot be disregarded (Jensen *et al.* 1994).

In summary, our results show that a gestational dose of 17 β -oestradiol (group E) is able to reproduce the changes in insulin sensitivity during pregnancy. However, the decrease in insulin sensitivity between days 11 and 16 is not associated with a decrease in *IRS-1* expression. We believe that this decrease in insulin sensitivity could be related to a modification in the intracellular distribution of *IRS-1*. Conversely, a pharmacological dose of 17 β -oestradiol mediated the development of insulin resistance through a decrease in *IRS-1* expression and protein phosphorylation in liver and adipose tissue. However, it is also possible that this could be related to a release of *IRS* proteins to the cytoskeletal scaffold. Moreover, the insulin resistance produced by the absence of female sex steroids is not related to changes in *IRS-1* expression.

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