Chronic 17 β -estradiol treatment improves skeletal muscle insulin signaling pathway components in insulin resistance associated with aging

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Abstract Insulin resistance is a common feature of aging in both humans and rats. In the case of females, it seems to be related to loss of gonadal function, due mainly due to a decrease in plasma estrogen levels. Several causes have been postulated for this insulin resistance, among them changes in several steps of the insulin pathway. In view of these findings, the purpose of the present study was to examine the role of chronic 17 β -estradiol treatment on insulin sensitivity during the aging process, and its effects on levels of the insulin-sensitive glucose transporter Glut4 (both total and plasma membrane localized), the interaction between p85 α subunit of PI3-k and IRS-1, Tyr- and Ser-612 phosphorylation of IRS-1 levels, and Ser-473 phosphorylation of Akt. The present findings indicate that 17 β -estradiol treatment is able to minimize the deleterious effect of aging on insulin sensitivity, at least at the level of plasma

membrane localized Glut4. Nevertheless further research is needed to determine this conclusively.

Keywords Aging · Insulin resistance · 17 β -estradiol · Glut4 · Akt, p85 α · IRS-1

Introduction

Part of the metabolic decline in aging is defined as the “syndrome of insulin resistance”, representing a group of metabolic defects that are important risk factors for age-related diseases (Barzilai et al. 2004). Since insulin is the main hormone implicated in carbohydrate metabolism regulation, insulin resistance can be considered as the hallmark of this metabolic worsening. This is a pathological state in which target tissues fail to respond to ordinary levels of insulin. It leads to a hormonal inability to provide normal glucose and lipid homeostasis (Mlinar et al. 2007).

Insulin resistance is a common feature in physiological conditions such as menopause and aging, but the underlying mechanisms are multifactorial (Reaven and Reaven 1985). It is obvious that, in addition to age, several factors may influence insulin sensitivity, including fat mass and its distribution, physical fitness, and genetic factors. When all these factors are taken into account, it is unclear whether age per se still has an independent effect on peripheral glucose uptake. However, in the case of females, insulin resistance seems to be related to a decrease in

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estrogen plasma levels caused by the loss of gonadal function (Alonso et al. 2006, 2008). These conditions could be determinant in the deleterious consequences of aging on insulin sensitivity.

At the cellular level, insulin action is mediated by a complex network of protein–protein interactions, mainly in skeletal muscle and adipose tissue. Insulin signaling transmission is initiated by binding of the hormone to its membrane receptor (IR), which activates the intrinsic protein tyrosine kinase activity of IR. This leads to autophosphorylation and subsequent phosphorylation of several interacting proteins including the insulin receptor substrate (IRS) family, which act as docking molecules for downstream signaling proteins containing SH2 domains, such as the regulatory subunit (85 kDa) of phosphatidylinositol 3-kinase (PI3-k), which in turn is responsible for the activation of the serine/threonine kinase Akt (also known as protein kinase B). Akt is activated by phosphorylation at two key residues: threonine 308 and serine 473. The IR/IRS-1/PI3K/Akt intracellular pathway is implicated in the translocation of the insulin-sensitive glucose transporter Glut4 to the plasma membrane to facilitate glucose transport into skeletal muscle cells and adipocytes. Although alterations in this signal transmission have been demonstrated, the molecular mechanism underlying insulin resistance with advanced age is still far from clear.

Interest in insulin pathway regulation has grown in recent years. Moreover, it has been shown that variation in Ser/Thr phosphorylation of IRS-1 acts as a physiological negative feedback control mechanism that uncouples IRS-1 from upstream and downstream effectors turning off insulin's transmission. Furthermore, this mechanism can be used by inducers of insulin resistance under pathological conditions. Thus, Ser/Thr phosphorylation could represent a generalized mechanism of insulin resistance (De Fea and Roth 1997; Herschkovitz et al. 2007).

Experimental studies (González et al. 2000, 2003; Kumagai et al. 1993) and clinical observations (Colacurci et al. 1998; Karjalainen et al. 2001) have demonstrated the importance of estrogen in the maintenance of insulin sensitivity in postmenopausal women. However, the route, dose and type of estrogen used appear to determine the efficacy of therapy. In this sense, the most important question is whether this type of therapy is a good tool against the inexorable consequences of aging in metabolic func-

tion in women, taking the risk/benefit ratio into account.

In recent years, several lines of evidence have accumulated indicating that not all of the physiological actions of estrogens can be explained by direct effects on gene transcription (classical effects), and the involvement of signaling pathways related to cytoplasmic proteins, growth factors and/or membrane-initiated responses has been reported (non-classical effects) (Levin 2005; Segars and Driggers 2002). In this sense, the molecular and cell-biological mechanisms underlying the metabolic action of estrogens are poorly understood, and further studies are required to elucidate these mechanisms.

In a former study, we showed that 17 β -estradiol treatment has beneficial effects on insulin sensitivity in aging rats (Alonso et al. 2006). In this work, we aimed to evaluate the effects of aging and chronic 17 β -estradiol treatment on proteins implicated in insulin signal transmission in the main insulin-sensitive tissue, i.e., skeletal muscle, with a view to finding out on what level of the insulin pathway estrogens might be acting.

Materials and methods

Animals

Virgin female Wistar rats (from the Biotery of the Faculty of Medicine, University of Oviedo) weighing 250–280 g (age 8–10 weeks), and kept under standard conditions of temperature (23 $\pm$ 3°C) and humidity (65 $\pm$ 1%), and a regular lighting schedule of 12 h light/dark cycle (0800–2000 hours) were used. The animals were fed with a standard diet (Panlab A04) and had free access to water. All experimental manipulations were performed between 0930 hours and 1230 hours. All experimental procedures carried out with animals were approved by a local veterinary committee from the University of Oviedo vivarium, and subsequent handling strictly followed the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Experimental design

Rats were ovariectomized through a midline incision under light anaesthesia by inhalation of halothane.

Ovariectomized rats were separated randomly into three groups: ovariectomized animals (O), ovariectomized animals treated with 17β -estradiol (E), and sham surgery animals (intact) (C), and were housed individually throughout the experiment.

Following surgery, all rats began the experimental treatment exactly 1 week after ovariectomy to ensure a uniform time of estrogen depletion before replacement, and to recover from surgery stress. After this, the rats were implanted subcutaneously in the posterior neck with 90-day-release 17β -estradiol pellets (25 μ g/pellet; Innovative Research of America, Sarasota, FL) or placebos containing no estradiol. The pellets were replaced every 90 days. This dosing regimen results in physiological levels of plasma estradiol and has been shown to be neuroprotective in rats (Harukuni et al. 2001).

Groups O, E and C were divided randomly into four subgroups (seven animals/subgroup): 6, 12, 18 and 24 (according to the month of the experimental period on which the animals were killed). Therefore, the animals were killed when they were approximately 8, 14, 20 and 26 months old. Moreover, 14 animals (7O and 7C) were sacrificed 1 week after ovariectomy (age 9–11 weeks). Therefore, the animals included in this group did not receive any treatment. These animals are considered as O–0 month and C–0 month groups.

The stage of the oestrous cycle in intact rats was determined by daily examination of fresh vaginal smears. Intact animals in diestrous phase were selected for subgroups 0 and 6. After month 12 of the experiment, none of the intact rats showed repetitive oestrous cycles; instead, 87.26% of animals showed persistent diestrous phase.

Euglycemic-hyperinsulinemic clamp

Clamp experiments were performed using a previously described procedure (González et al. 2000), the results of which were published recently (Alonso et al. 2006). Before and after the clamp study, blood samples for the determination of insulin and 17β -estradiol plasma concentrations were collected from the jugular vein into heparinized tubes, centrifuged at 3,000 rpm for 20 min at 4°C and plasma was immediately drawn off and stored frozen at –20°C until assayed. Plasma insulin was measured by radioimmunoassay (RIA) using a DGR Instruments

(Marburg, 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 Immunchen kits of cover tubes (ICN Biomedicals, Barcelona, Spain). The assay sensitivity was 10 pg/ml, and the intra-assay coefficient of variation was 9.45%. All samples were measured on the same day. Samples were assayed in triplicate.

Crude extracts and plasma membrane preparation

After clamp experiments, samples of skeletal muscle (flexor digitorum superficialis, extensor digitorum longus, soleus and extensor digitorum lateralis) were quickly removed and homogenized in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.05% sodium deoxycholate, sodium orthovanadate, 5 mM EDTA, 10% glycerol) at 4°C. The extracts were centrifuged at 21,000 g at 4°C for 10 min and the protein content of the supernatant was determined by the Bradford dye-binding method (Bradford 1976). Cell membrane preparations from skeletal muscle were prepared by a modification of the method described by Hirshman et al. (1990). Briefly, a total of 500 mg skeletal muscle was homogenized with a Polytron operated at maximum speed for 30 s at 4°C in a buffer containing 100 mM Tris (pH 7.5), 20 mM EDTA (pH 8.0) and 255 mM sucrose (pH 7.6). The homogenate was then centrifuged at 1,000 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 Na^+ - K^+ -ATPase. The membrane fraction was prepared by resuspending the pellet in 20 mM HEPES and 250 mM sucrose, pH 7.4 (buffer A). 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 A. The resulting interface and the entire buffer above it were collected, diluted in an equal amount of buffer A, and centrifuged 1 h at 227,000 g. The resulting pellet was used as the membrane fraction; its protein content was determined as described above using the Bradford dye-binding method.

Immunoprecipitation and western-blot analysis

Firstly, proteins in the crude homogenate were resolved by SDS-PAGE (10% Tris-Acrylamide-Bis) and electrotransferred from the gel to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia, Piscataway, NJ) as described by Towbin et al. (1979). Non-specific protein binding to the nitrocellulose membrane was reduced by preincubating the filter in blocking buffer (TNT, 7% BSA), and the membrane was then incubated overnight with the primary antibody. Antibodies against Glut4 (sc-7938, diluted 1:2,500), Akt (sc-7126, diluted 1:2,000) and Ser 473 Akt (sc-101629, diluted 1:2,500) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). After incubation with the primary antibody, the membranes were washed and incubated with an antirabbit antibody coupled to HRP (sc-2004 Santa Cruz, diluted 1:20,000), or an antigoat antibody coupled to HRP (sc-2768 Santa Cruz, diluted 1:20,000) respectively. Finally, all membranes were stripped and probed with a monoclonal anti- β -actin antibody (sc-1615 Santa Cruz, diluted 1:2,500). Immunoreactive bands were detected using an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). Films were analyzed using a digital scanner (Nikon AX-110) and NIH Image 1.57 software. The density of each band was normalized to its respective loading control (β -actin) and represented as a percentage of control values (intact rats of 0 month, group C0). In order to minimize inter-assay variations, in each experiment samples from all animal groups were processed in parallel.

The same protocol was performed to determine Glut4 protein content in the plasma membrane. To test the purity of plasma membrane fractions, membranes were incubated in stripping buffer and another Western-blot analysis was performed using anti- Na^+/K^+ -ATPase $\alpha 1$ subunit antibody (sc-16041 Santa Cruz Biotech, diluted 1:5,000). After incubation with the primary antibody, the membranes were washed and incubated with an antigoat antibody coupled to HRP (sc-2768 Santa Cruz, diluted 1:20,000).

Aliquots containing 500 μg protein from the crude homogenate were subjected to immunoprecipitation using polyclonal antibodies against IRS-1 (sc-559 Santa Cruz, diluted 1:10,000). Immunoprecipitations were performed using a non-immune rabbit serum to

verify the specificity of the bands detected by Western blotting. The immune complexes were adsorbed and precipitated using Protein G-agarose beads (sc-2002, Santa Cruz) overnight at 4°C in a rocking platform, after which they were washed several times in lysis buffer, 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 denatured in a boiling water bath for 5 min. The immunoprecipitates were resolved by 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Western blotting was performed as described above using an antibody against the p85 α subunit of PI3-k (sc-1637 Santa Cruz, diluted 1:10,000), against the phosphotyrosine (sc-7020, diluted 1:2,500), obtained from Santa Cruz Biotechnologies (Santa Cruz, CA), or against phosphoserine 612 IRS-1 (2386S, diluted 1:5,000) obtained from Cell Signaling Technology (Danvers, MA).

Statistical analysis

Data are expressed as mean $\pm$ standard error of mean (SEM). First, we evaluated the Gaussian distribution of each variable. Thereafter, data were analyzed statistically using an ANOVA design followed by between-group comparisons using the Tukey honestly significant difference (HSD) test. The data for month 0 were analyzed by impaired Student's *t* testing. Values of $P \leq 0.05$ were considered significant. Statistical analysis was performed using SPSS v.14.0.1 for Windows.

Results

Table 1 shows body weight, fasting blood glucose, fasting serum insulin and serum insulin after the clamp experiment. Body weight was significantly higher in group O compared to groups C and E, and higher in C than in E except at time 0 of the experiment. On the other hand, an increase in body weight throughout the study was observed in all groups. Fasting blood glucose levels were observed to be similar between groups and between months of the experimental period. Although there was a tendency towards increased fasting serum insulin throughout the experimental period in group E, no significant changes were found, whereas groups C and O displayed a significant increase from the start to the end of the experiment. On

Table 1 Body weight, fasting blood glucose, fasting serum insulin and serum insulin after clamp experiments of ovariectomized animals (O), sham surgery animals (intact) (C) andovariectomized animals treated with 17 β -estradiol (E). Mean $\pm$ standard error of the mean for seven animals

		0 Months	6 Months	12 Months	18 Months	24 Months
Body weight (g)	C	276.26 $\pm$ 3.49 *	321.71 $\pm$ 8.45 *	350.59 $\pm$ 7.56 *	382.59 $\pm$ 9.65 *	392.48 $\pm$ 6.79
	O	274.06 $\pm$ 2.85 *	355.67 $\pm$ 9.00 *	379.00 $\pm$ 7.66 *	457.6 $\pm$ 12.89 *	488.33 $\pm$ 7.80
	E	279.5 $\pm$ 1.97 *	310.86 $\pm$ 8.78 *	345.4 $\pm$ 8.77 *	363.43 $\pm$ 14.39 *	370.67 $\pm$ 8.16
Intramonth comparisons			O vs C,E	O vs C,E	C vs O, E; O vs E	C vs O, E; O vs E
Fasting blood glucose (mg/dl)	C	94.26 $\pm$ 3.26	93.70 $\pm$ 3.62	92.53 $\pm$ 2.71	94.65 $\pm$ 4.21	95.55 $\pm$ 3.16
	O	94.23 $\pm$ 3.17	92.66 $\pm$ 4.52	90.50 $\pm$ 5.38	95.20 $\pm$ 4.40	97.16 $\pm$ 3.89
	E	95.25 $\pm$ 4.23	88.00 $\pm$ 4.02	96.80 $\pm$ 9.72	99.71 $\pm$ 4.27	93.16 $\pm$ 3.49
Fasting serum insulin (ng/ml)	C	0.95 $\pm$ 0.10	1.55 $\pm$ 0.12	1.95 $\pm$ 0.20 *	2.87 $\pm$ 0.13 *	3.45 $\pm$ 0.27
	O	0.91 $\pm$ 0.12 *	3.74 $\pm$ 0.18 *	2.04 $\pm$ 0.12 *	3.60 $\pm$ 0.16 *	3.29 $\pm$ 0.15
	E	0.90 $\pm$ 0.06	0.97 $\pm$ 0.03	1.37 $\pm$ 0.13	1.46 $\pm$ 0.12	1.60 $\pm$ 0.10
Intramonth comparisons			O vs C,E	O vs C,E	O vs C,E; C vs E	E vs C,O
Serum insulin after clamp experiments (ng/ml)	C	6.21 $\pm$ 0.30 *	9.98 $\pm$ 0.7 *	11.25 $\pm$ 1.35 *	15.83 $\pm$ 1.23 *	18.74 $\pm$ 1.21
	O	5.98 $\pm$ 0.63 *	15.01 $\pm$ 0.50 *	18.03 $\pm$ 0.61 *	26.70 $\pm$ 0.67 *	29.78 $\pm$ 0.84
	E	5.73 $\pm$ 0.84 *	12.42 $\pm$ 0.25 *	15.44 $\pm$ 0.69 *	21.31 $\pm$ 0.96 *	24.01 $\pm$ 0.54
Intramonth comparisons			C vs O, E; O vs E	C vs O, E; O vs E	C vs O, E; O vs E	C vs O, E; O vs E

* $P \leq 0.05$ (month vs next month)

the other hand, fasting serum insulin was always significantly higher in O than in C and E, except at month 24. Finally, after clamp experiments, we found a significant increase in serum insulin levels throughout experimental period in all groups. Moreover, this parameter was always significantly higher in O than in C and E, and significantly higher in E than in C.

Plasma 17 β -estradiol values obtained during the study are shown in Fig. 1. The plasma level of

estradiol was significantly higher in groups E and C than in group O at all time points. In addition, plasma levels of 17 β -estradiol were significantly higher in group E than in group C at 18 and 24 months. In the C group animals, plasma levels of 17 β -estradiol increased significantly at 6 months and then decreased significantly at 24 months. In the E group, the estradiol level did not change significantly during the study. A significant decrease in the estradiol level was found in group O during the first 6 months.

To investigate insulin resistance in rats at different months of the hormonal treatment, we carried out glucose clamp experiments under euglycemic-hyperinsulinemic conditions (DeFronzo et al. 1979). Figure 2 shows the results of the clamp experiments. In intact rats (C), a significant increase in insulin sensitivity was noted until 12 months, followed by a significant decrease until the end of the study, while this parameter diminished significantly in both groups E and O from 6 months until the end of the experimental period. Analyzing intergroup differences, we observed that group C showed higher insulin sensitivity than the other groups, and this parameter was always the lowest in the O group.

With respect to total Glut4 levels (Fig. 3), similar patterns were found in groups C and E; there was a

Fig. 1 Levels of 17 β -estradiol of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with 17 β -estradiol (E; gray bars). Mean $\pm$ standard error of the mean for seven animals. Significant differences are shown. * $P \leq 0.05$ (month vs next month)

Fig. 2 Comparison of glucose infusion rates of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with 17 β -estradiol (E; gray bars). Glucose infusion rate was assessed as the mean values from 40 to 60 min during euglycemic-hyperinsulinemic clamp experiments. Mean $\pm$ standard error of the mean for seven animals. Significant differences are shown. * $P \leq 0.05$ (month vs next month)

significant increase between 0 and 6 months and between 18 and 24 months, and a significant decrease between 6 and 12 months, respectively. In group O, the results were different since the total amount of Glut4 decreased significantly between 0 and 6 months and between 18 and 24 months, and increased significantly between 6 and 12 months, respectively.

The amount of total Glut4 at months 6 and 24 was significantly higher in groups C and E than in group O. However, at 12 and 18 months of treatment, C group showed the lowest Glut4 total protein content.

Figure 4 represents the Glut4 protein content localized in the plasma membrane. Again, groups C and E showed similar patterns, i.e., a significant increase until 12 months followed by a significant decrease to the end of the experimental period. On the contrary, in group O, Glut4 protein content decreased from the start to the end of the study. When the differences between groups at different times were examined, it was found that this parameter is higher in C group than in the others, except at 24 months, when Glut4 plasma membrane content was similar in groups C and E.

In order to study the intracellular insulin signaling pathway, we checked the first steps in this pathway, and found no significant intra- or inter-group differences in relation to the absolute protein abundance of IRS-1, p85 α , Akt, insulin receptor and insulin receptor phosphorylation (data not shown).

Immunoprecipitation studies were performed in order to evaluate the interaction between p85 α (regulatory subunit of PI3-k) and IRS-1 (Fig. 5). With respect to this parameter, similar patterns were found in all groups; there was a significant increase until

Fig. 3 Glut4 total protein content of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with 17 β -estradiol (E; gray bars). The histogram shows the densitometric analysis of the

western blots. Values are means $\pm$ SEM ($n=7$), and represented as a percentage of control values (rats of 0 month from group C). Only significant differences are shown. * $P \leq 0.05$ (month vs next month)

Fig. 4 Glut4 plasma membrane protein content of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with 17 β -estradiol (E; gray bars). The histogram shows the densitometric

analysis of the western blots. Values are means $\pm$ SEM ($n=7$), and represented as a percentage of control values (rats of 0 month from group C). Only significant differences are shown. * $P \leq 0.05$ (month vs next month)

12 months, followed by a significant decrease to the end of the study. However, when differences between groups were assessed, it was observed that this interaction is significantly higher in group C than in

the others, revealing group E to have the lowest p85 α -IRS-1 interaction, except at month 24.

In addition, changes in tyrosine phosphorylation of IRS-1 during the aging process were assessed (Fig. 6).

Fig. 5 Association between p85 α and insulin receptor substrate 1 (IRS-1) of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with 17 β -estradiol (E; gray bars). The histogram

shows the densitometric analysis of the western blots. Values are means $\pm$ SEM ($n=7$), and represented as a percentage of control values (rats of 0 month from group C). Only significant differences are shown. * $P \leq 0.05$ (month vs next month)

Fig. 6 IRS-1 tyrosine phosphorylation of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with 17 β -estradiol (E; gray bars). The histogram shows the densitometric

analysis of the western blots. Values are means $\pm$ SEM ($n=7$), and represented as a percentage of control values (rats of 0 month from group C). Only significant differences are shown. * $P\leq 0.05$ (month vs next month)

All experimental groups showed a similar pattern of this parameter throughout the study, increasing significantly until 12 months followed by a significant decrease from 12 to 24 months, except in group E, which exhibited no change between 6 and 12 months. On the other hand, IRS-1 tyrosine phosphorylation was significantly greater in group E than in the others at 6 and 18 months. Group O had the lowest tyrosine phosphorylation level, except at 24 months when it was the highest.

Figure 7 illustrates changes in serine 612 phosphorylation of IRS-1 during aging. The temporal pattern of this parameter was similar in the three groups studied: a significant increase was noted until 18 months, followed by a significant decrease to the end of the study. Group E reached the highest level of IRS-1 serine 612 phosphorylation at 12 months, with group O being highest at 18 months.

Ser-473 Akt phosphorylation rates are shown in Fig. 8. The temporal pattern of this parameter differed in the three groups studied. In groups C and E, we observed a significant increase until 12 months. However, while in group C a significant decrease was noted until month 24, no significant change was seen in group E. In group O there was a clearly tendency for Ser-473 Akt phosphorylation to decrease

throughout experiment, but significant differences was noted only between 18 and 24 months. At months 0 and 6, no differences between experimental groups in relation to Ser-473 Akt phosphorylation were seen. However, when differences between groups were determined at 12, 18 and 24 months, it was observed that Ser-473 Akt phosphorylation is significantly higher in group E than in the others, with group C showing the lowest phosphorylation rate, except at month 12.

Discussion

Insulin resistance is manifested by decreased insulin-stimulated glucose uptake and metabolism in target tissues and by impaired suppression of hepatic glucose output (Reaven and Reaven 1985). These functional defects may result, at least in part, from down-regulation of the major insulin-responsive glucose transporter, Glut4. Since muscle has long been considered the main site of insulin-stimulated glucose uptake in vivo, with adipose tissue contributing relatively little to total body glucose disposal (DeFronzo et al. 1981), we focus on the Glut4 transporter in muscle.

Fig. 7 IRS-1 Ser-612 phosphorylation of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with 17 β -estradiol (E; gray bars). The histogram shows the densitometric

analysis of the western blots. Values are means $\pm$ SEM ($n=7$), and represented as a percentage of control values (rats of 0 month from group C). Only significant differences are shown. * $P \leq 0.05$ (month vs next month)

The changes observed in Glut4 total protein in skeletal muscle from aging rats do not explain the observed worsening of insulin sensitivity, because the pattern of this protein differs from the clamp results.

In this sense the highest sensitivity throughout the experimental period is reached by the intact group (C), although this group does not exhibit the highest Glut4 total amount. Consistent with these findings,

Fig. 8 Akt Ser-473 phosphorylation of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with 17 β -estradiol (E; gray bars). The histogram shows the densitometric analysis of the

western blots. Values are means $\pm$ SEM ($n=7$), and represented as the percent of control values (rats of 0 month from group C). Only significant differences are shown. * $P \leq 0.05$ (month vs next month)

we hypothesized that changes in insulin sensitivity during aging could not be explained by variations in the total amount of Glut4. The temporal pattern of the Glut4 total amount (Fig. 3) is similar in groups C and E but different in group O, thus implicating 17 β -estradiol as one of the factors possibly involved in controlling Glut4 total protein content during aging.

Considering that Glut4 translocation from an intracellular pool to the plasma membrane is required for effective glucose uptake, the next step in our study was to evaluate the amount of this transporter in the plasma membranes of skeletal muscle tissue. This metabolic process is impaired in individuals with insulin resistance, where skeletal muscle fails to respond to physiological levels of insulin (Kahn and Flier 2000). In agreement with findings in the literature, our results show that plasma membrane Glut4 diminished from 12 months to the end of the experimental period in the three studied groups (Fig. 4), which also corroborates our clamp results. In the light of these results, we could infer a possible role for 17 β -estradiol in Glut4 translocation during aging, i.e., age-associated absence of estradiol could result in impairment of Glut4 translocation to the plasma membrane, thus leading to insulin resistance. This idea is in line with that of Rincon et al. (1996), who reported that the absence of female sex hormones, as well as testosterone treatment of oophorectomized female rats results in decreased whole-body insulin-mediated glucose uptake.

Moreover, 17 β -estradiol might favor mobilization of Glut4-containing vesicles to the cell surface. Compelling evidence for this scenario exists—a Glut4 sequence implicated in Glut4 transport from the cell surface to a subdomain of the trans-Golgi network enriched in the t-SNAREs Syntaxins 6 and 16 has been reported (Shewan et al. 2003). To date, no relationship between sex hormones and these molecules has been reported in insulin-dependent tissues, although it has been shown in α T3-1 cells that estradiol regulates expression and content of munc-18 (Weiss et al. 2007), a protein involved in Glut4 mobilization (D'Andrea-Merrins et al. 2007). Taken together, this again might indicate a possible role for 17 β -estradiol in Glut4 translocation.

The results presented here show that 17 β -estradiol increases phosphorylation of Akt during the second half of the experiment in group E (Fig. 8), as has also been shown recently by others (Vasconsuelo et al.

2008). This might suggest a potential beneficial effect of estrogen replacement therapy on the insulin resistance associated with the aging process, because it may stimulate glucose uptake by skeletal muscle. However, we observed no increase in plasma membrane Glut4 content in relation to group C. These results are in agreement with those of Rogers et al. (2009), who demonstrated recently that estradiol stimulates Akt in intact skeletal muscle but does not stimulate muscle glucose uptake. The gradual drop in Akt phosphorylation in group C throughout the experiment seems to suggest not only that the loss of insulin sensitivity associated with aging could be related to decreased levels of Glut4 in the plasma membrane, but also that other ovarian hormones, e.g., progesterone, could participate in the regulation of Akt serine phosphorylation in skeletal muscle.

Since age-associated impairment of insulin sensitivity, in which estradiol could play an important role, seems to involve decreased translocation of Glut4 transporters to the plasma membrane, the next step in our study was designed to evaluate signaling molecules, such as the p85 α –IRS-1 association, implicated in insulin transmission. PI3-k activation through recruitment of tyrosine phosphorylated sites of IRS-1 is a key step in the Glut4 mobilization induced by insulin (Czech and Corvera 1999; Kahn and Flier 2000). It is likely that a reduction in the p85 α –IRS-1 interaction might be essential to the insulin resistance associated with aging. The fact that we found no significant intra- or inter-group differences in the absolute protein abundance of IRS-1, p85 α , insulin receptor or insulin receptor phosphorylation (data not show) could reasonably explain our results (Fig. 5) because, in all three experimental groups, there was a significant increase in p85 α –IRS-1 interaction until 12 months, followed by a significant decrease to the end of the study, supporting the stated hypothesis. This evidence is compatible with that found by other studies, e.g., that of Carvalho et al. (1996). The early loss of ovarian function seems to be responsible for a decrease in the p85 α –IRS-1 association, and estradiol treatment does not seem to be able to compensate for this. This interaction is even lower between months 6 and 18 in group E compared to group O. We believe that other factors, e.g., progesterone and other hormones, could be responsible for control of the p85 α –IRS-1 association. However, we conclude that this interaction is one of the most important inter-

mediates in several intracellular signaling pathways. Therefore, we cannot discount the possibility that the present results can be considered from several different points of view. However, they seem to demonstrate that the p85 α –IRS-1 association represents an important complex that is modulated by the aging process.

The temporal pattern of this association in groups C and E is similar to that observed in Glut4 localized in the plasma membrane. In light of these results we can infer that the p85 α –IRS-1 association influences the amount of transporter localized in the plasma membrane in groups C and E during the aging process. However, the seeming paradox that the p85 α –IRS-1 interaction increased up to 12 months in O group and decline thereafter, while Glut4 plasma membrane protein content exhibited a different pattern, led us to ask if the absence of estradiol during aging would affect the insulin signaling pathway downstream of the p85 α –IRS-1 association. As we have commented above, 17 β -estradiol could play a critical role at the level of Glut4 translocation, and its absence might lead to defects in glucose uptake during the aging process.

In searching for a feasible hypothesis that would support the decrease in p85 α –IRS-1 interplay during aging, the next logical step in our study was to evaluate the level of IRS-1 tyrosine phosphorylation, since, when phosphorylated, these sites as well as others appear to be involved in the non-covalent interaction of IRS-1 with the regulatory subunit of PI3-k (p85 α). Indeed, our results showed a decrease in IRS-1 tyrosine phosphorylation from 12 months onwards in the three studied groups (Fig. 6). Thus, a reduction in IRS-1 tyrosine phosphorylation could be involved in the decline in p85 α –IRS-1 association observed during aging, which might lead to age-related insulin resistance. Our results are in line with an earlier report showing a progressive reduction in IRS-1 tyrosine phosphorylation in response to insulin during aging (Carvalho et al. 1996).

On the other hand, compared to group O, estradiol treatment markedly increased levels of IRS-1 tyrosine phosphorylation, except at 24 months. Therefore, these results suggest that estradiol controls tyrosine phosphorylation of IRS-1, and appears to prevent the effects not only of ovariectomy but also of the aging process, at least until 18 months. These results reveal another level of insulin signaling transmission at

which estradiol could improve insulin sensitivity during aging.

As has already been extensively documented, the p85 α –IRS-1 association involves not only IRS-1 tyrosine phosphorylation but also phosphorylation of serine. Current data support the hypothesis that serine phosphorylation at site 612, next to PI3-k joining residues, negatively regulates the p85 α –IRS-1 interaction (Delahaye et al. 1998). In light of this evidence, the rate of Ser-612 phosphorylation of IRS-1 was examined in order to infer its possible effect on p85 α –IRS-1 interaction during the aging process.

We found that Ser-612 phosphorylation in IRS-1 increased until 18 months in the three studied groups, with a notable decrease thereafter. Taken together, this could indicate that there is an increase in the Ser-612 phosphorylation level associated with the aging process, which could be one of the factors responsible for insulin resistance during aging.

Several lines of evidence suggest that fatty acid uptake is significantly increased in old animals, which may contribute to the accumulation of triglycerides in the muscle of old animals (Tucker and Turcotte 2003). This fact has been proposed as one of the main reasons why insulin resistance develops during aging. Randle et al. (1963) were the first to suggest that fatty acids could induce insulin resistance in skeletal muscle, showing that fatty acids compete with glucose for substrate oxidation. At a molecular level, it has been proposed that the link between fatty acids and insulin resistance could be inhibition of the insulin network by phosphorylation at serine residues catalyzed by the enzyme PKC (Yu et al. 2002). In this sense, cell incubation with PKC activators is associated with increased Ser-612 phosphorylation (De Fea and Roth 1997). Given these data, we suspect that the increase in this parameter in the three experimental groups could be due to the rise in fatty acid uptake associated with aging. However, a decrease in this parameter was found in the last time period of the study. It is likely that, at this point, phosphorylation of other sites could take place, contributing to the observed insulin resistance. Phosphorylation of other residues could have a negative feedback function and thus desensitize insulin signaling (White 2006). Therefore, increased knowledge of the regulation of IRS-1 Ser/Thr phosphorylation could lead to a better understanding of insulin signaling and insulin resistance.

On the other hand, no between-group differences in Ser-612 phosphorylation levels were detected. Therefore, phosphorylation in this motif appears not to be controlled exclusively by estradiol; other metabolic and/or hormonal parameters might play an important role in the regulation of IRS-1 Ser-612 phosphorylation.

To summarize, the 12-month timepoint of the experimental period appeared to represent a break-even stage in group C animals, both in terms of insulin sensitivity, and plasma membrane Glut4, p85 α -IRS-1 association and IRS-1 tyrosine phosphorylation. This coincides with the time at which intact animals showed irregular cycling, mainly during persistent diestrous phase. In this sense, this timepoint might be considered as the beginning of the impairment in ovulatory function and altered patterns of steroid secretion (Anzalone et al. 2001; Matt et al. 1987; Nass et al. 1984).

In the light of our results, we can infer that, during aging, 17 β -estradiol treatment improves glucose homeostasis in the absence of this sexual steroid, mainly at the level of Glut4, because the amounts of this transporter localized in plasma membrane of skeletal muscle increased significantly compared with levels in group O. Nevertheless, further research is needed to determine this conclusively.

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References

- Alonso A, Fernández R, Moreno M, Ordóñez P, González-Pardo H, Conejo NM, Díaz F, González C (2006) Positive effects of 17 β -estradiol on insulin sensitivity in aged ovariectomized female rats. *J Gerontol A Biol Sci Med Sci* 61:419–426
- Alonso A, Moreno M, Ordóñez P, Fernández R, Pérez C, Díaz F, Navarro A, Tolivia J, González C (2008) Chronic estradiol treatment improves brain homeostasis during aging in female rats. *Endocrinology* 149:57–72. doi:10.1210/en.2007-0627
- Anzalone CR, Hong LS, Lu JK, LaPolt PS (2001) Influences of age and ovarian follicular reserve on estrous cycle patterns, ovulation, and hormone secretion in the Long-Evans rat. *Biol Reprod* 64:1056–1062. doi:10.1095/biolreprod64.4.1056
- Barzilai N, Rossetti L, Lipton RB (2004) Einstein's Institute for Aging Research: collaborative and programmatic approaches in the search for successful aging. *Exp Gerontol* 39:151–157. doi:10.1016/j.exger.2003.10.009
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254. doi:10.1016/0003-2697(76)90527-3
- Carvalho CR, Brenelli SL, Silva AC, Nunes AL, Velloso LA, Saad MJ (1996) Effect of aging on insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of rats. *Endocrinology* 137:151–159. doi:10.1210/en.137.1.151
- Colacurci N, Zarcone R, Mollo A, Russo G, Passaro M, de Seta L, de Franciscis P (1998) Effects of hormone replacement therapy on glucose metabolism. *Panminerva Med* 40:18–21
- Czech MP, Corvera S (1999) Signaling mechanisms that regulate glucose transport. *J Biol Chem* 274:1865–1868. doi:10.1074/jbc.274.4.1865
- D'Andrea-Merrins M, Chang L, Lam AD, Ernst SA, Stuenkel EL (2007) Munc18c interaction with syntaxin4 monomers and SNARE complex intermediates in GLUT4 vesicle trafficking. *J Biol Chem* 282:16553–16566. doi:10.1074/jbc.M610818200
- De Fea K, Roth RA (1997) Protein kinase C modulation of insulin receptor substrate-1 tyrosine phosphorylation requires serine 612. *Biochemistry* 36:12939–12947. doi:10.1021/bi971157f
- DeFronzo RA, Tobin JD, Andres R (1979) Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237:E214–E223
- DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP (1981) The effect of insulin on the disposal of intravenous glucose. *Diabetes* 30:1000–1007
- Delahaye L, Mothe-Satney I, Myers MG, White MF, Van Obberghen E (1998) Interaction of insulin receptor substrate-1 (IRS-1) with phosphatidylinositol 3-kinase: effect of substitution of serine for alanine in potential IRS-1 serine phosphorylation sites. *Endocrinology* 139:4911–4919. doi:10.1210/en.139.12.4911
- González C, Alonso A, Alvarez N, Díaz F, Martínez M, Fernández S, Patterson AM (2000) Role of 17 β -estradiol and/or progesterone on insulin sensitivity in the rat: implications during pregnancy. *J Endocrinol* 166:283–291. doi:10.1677/joe.0.1660283
- González C, Alonso A, Díaz F, Patterson AM (2003) Dose- and time-dependent effects of 17 β -oestradiol on insulin sensitivity in insulin-dependent tissues of rat: implications of IRS-1. *J Endocrinol* 176:367–379. doi:10.1677/joe.0.1760367
- Harukuni I, Hurn PD, Crain BJ (2001) Deleterious effect of beta-estradiol in a rat model of transient forebrain ischemia. *Brain Res* 900:137–142. doi:10.1016/S0006-8993(01)02278-8
- Herschkovitz A, Liu YF, Ilan E, Ronen D, Boura-Halfon S, Zick Y (2007) Common inhibitory serine sites phosphorylated by IRS-1 kinases, triggered by insulin and inducers of insulin resistance. *J Biol Chem* 282:18018–18027. doi:10.1074/jbc.M610949200
- Hirshman MF, Goodyear LJ, Wardzala LJ, Horton ED, Horton ES (1990) Identification of an intracellular pool of glucose transporters from basal and insulin-stimulated rat skeletal muscle. *J Biol Chem* 265:987–991
- Kahn BB, Flier JS (2000) Obesity and insulin resistance. *J Clin Invest* 106:473–481. doi:10.1172/JCI10842

- Karjalainen A, Paasilta M, Heikkinen J, Bäckström AC, Savolainen M, Kesäniemi YA (2001) Effects of peroral and transdermal oestrogen replacement therapy on glucose and insulin metabolism. *Clin Endocrinol* 54:165–173. doi:[10.1046/j.1365-2265.2001.01208.x](https://doi.org/10.1046/j.1365-2265.2001.01208.x)
- Kumagai S, Holmång A, Björntorp P (1993) The effects of oestrogen and progesterone on insulin sensitivity in female rats. *Acta Physiol Scand* 149:91–97. doi:[10.1111/j.1748-1716.1993.tb09596.x](https://doi.org/10.1111/j.1748-1716.1993.tb09596.x)
- Levin ER (2005) Integration of the extranuclear and nuclear actions of estrogen. *Mol Endocrinol* 19:1951–1959. doi:[10.1210/me.2004-0390](https://doi.org/10.1210/me.2004-0390)
- Matt DW, Sarver PL, Lu JK (1987) Relation of parity and estrous cyclicity to the biology of pregnancy in aging female rats. *Biol Reprod* 37:421–430. doi:[10.1095/biolreprod37.2.421](https://doi.org/10.1095/biolreprod37.2.421)
- Mlinar B, Marc J, Janez A, Pfeifer M (2007) Molecular mechanisms of insulin resistance and associated diseases. *Clin Chim Acta* 375:20–35. doi:[10.1016/j.cca.2006.07.005](https://doi.org/10.1016/j.cca.2006.07.005)
- Nass TE, LaPolta PS, Judd HL, Lu JK (1984) Alterations in ovarian steroid and gonadotrophin secretion preceding the cessation of regular oestrous cycles in ageing female rats. *J Endocrinol* 100:43–50. doi:[10.1677/joe.0.1000043](https://doi.org/10.1677/joe.0.1000043)
- Randle PJ, Garland PB, Hales CN, Newsholme EA (1963) The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1:785–789. doi:[10.1016/S0140-6736\(63\)91500-9](https://doi.org/10.1016/S0140-6736(63)91500-9)
- Reaven GM, Reaven EP (1985) Age, glucose intolerance, and non-insulin-dependent diabetes mellitus. *J Am Geriatr Soc* 33:286–290
- Rincon J, Holmang A, Wahlstrom EO, Lonnroth P, Björntorp P, Zierath JR, Wallberg-Henriksson H (1996) Mechanisms behind insulin resistance in rat skeletal muscle after oophorectomy and additional testosterone treatment. *Diabetes* 45:615–621. doi:[10.2337/diabetes.45.5.615](https://doi.org/10.2337/diabetes.45.5.615)
- Rogers NH, Witczak CA, Hirshman MF, Goodyear LJ, Greenberg AS (2009) Estradiol stimulates Akt, AMP-activated protein kinase (AMPK) and TBC1D1/4, but not glucose uptake in rat soleus. *Biochem Biophys Res Commun* 382:646–650. doi:[10.1016/j.bbrc.2009.02.154](https://doi.org/10.1016/j.bbrc.2009.02.154)
- Segars JH, Driggers PH (2002) Estrogen action and cytoplasmic signaling cascades. Part I: membrane-associated signaling complexes. *Trends Endocrinol Metab* 13:349–354. doi:[10.1016/S1043-2760\(02\)00633-1](https://doi.org/10.1016/S1043-2760(02)00633-1)
- Shewan AM, van Dam EM, Martin S, Luen TB, Hong W, Bryant NJ, James DE (2003) GLUT4 recycles via a trans-Golgi network (TGN) subdomain enriched in Syntaxins 6 and 16 but not TGN38: involvement of an acidic targeting motif. *Mol Biol Cell* 14:973–986. doi:[10.1091/mbc.E02-06-0315](https://doi.org/10.1091/mbc.E02-06-0315)
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354. doi:[10.1073/pnas.76.9.4350](https://doi.org/10.1073/pnas.76.9.4350)
- Tucker MZ, Turcotte LP (2003) Aging is associated with elevated muscle triglyceride content and increased insulin-stimulated fatty acid uptake. *Am J Physiol* 285:E827–E835
- Vasconsuelo A, Milanese L, Boland R (2008) 17Beta-estradiol abrogates apoptosis in murine skeletal muscle cells through estrogen receptors: role of the phosphatidylinositol 3-kinase/Akt pathway. *J Endocrinol* 196:385–397. doi:[10.1677/JOE-07-0250](https://doi.org/10.1677/JOE-07-0250)
- Weiss JM, Huller H, Polack S, Friedrich M, Diedrich K, Treeck O, Pfeiler G, Ortmann O (2007) Estradiol differentially modulates the exocytotic proteins SNAP-25 and munc-18 in pituitary gonadotrophs. *J Mol Endocrinol* 38:305–314. doi:[10.1677/jme.1.02114](https://doi.org/10.1677/jme.1.02114)
- White MF (2006) Regulating insulin signaling and beta-cell function through IRS proteins. *Can J Physiol Pharmacol* 84:725–737. doi:[10.1139/Y06-008](https://doi.org/10.1139/Y06-008)
- Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI (2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277:50230–50236. doi:[10.1074/jbc.M200958200](https://doi.org/10.1074/jbc.M200958200)