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ENDURANCE EXERCISE TRAINING INCREASES INSULIN RESPONSIVENESS IN ISOLATED ADIPOCYTES THROUGH IRS/PI3-KINASE/AKT PATHWAY.

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Running Head: Physical training and adipocyte insulin signaling

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Abstract

Endurance exercise training promotes important metabolic adaptations and the adipose tissue is particularly affected. The aim of this study was to investigate how endurance exercise training modulates some aspects of insulin action in isolated adipocytes and in intact adipose tissue. Male Wistar rats were submitted to daily treadmill running (1 hour per day), for 7 weeks. Sedentary age-matched rats were used as controls. Final body weight, body weight gain and epididymal fat pad weight did not show any statistical differences between the groups. Adipocytes from trained rats were smaller than those from sedentary rats (205 ± 16.8 vs. 286 ± 26.4 pl, $p<0.05$). Trained rats showed decreased plasma glucose (4.9 ± 0.13 vs. 5.3 ± 0.07 mmol/l, $p<0.05$), and insulin levels (0.24 ± 0.012 vs. 0.41 ± 0.049 mmol/l, $p<0.05$) and increased insulin-stimulated glucose uptake (23.1 $\pm$ 3.1 vs. 12.1 $\pm$ 2.9 pmol/cm^2, $p<0.05$) when compared to sedentary rats. The number of insulin receptors and the insulin-induced tyrosine phosphorylation (PY) of IR-β subunit did not change between the groups. Insulin-induced PY-IRS-1 and -2 increased significantly (1.57- and 2.38-fold, respectively) in trained rats. Insulin-induced IRS-1/PI3-kinase (but not IRS-2/PI3-K) association and serine AKT phosphorylation also increased (2.06- and 3.15-fold, respectively) after training. The protein content of IR-β subunit, IRS-1 and -2 did not differ between the groups. Taken together, these data support the hypothesis that the increased adipocyte responsiveness to insulin observed after endurance exercise training is modulated by IRS/PI3-K/AKT pathway.

Key words: adipose tissue, insulin signaling, exercise, diabetes and obesity

INTRODUCTION

Endurance exercise training has been recognized as an important therapeutic tool used to treat and prevent obesity and type II diabetes mellitus, diseases which are characterized by an increased insulin resistance leading to a diminished peripheral glucose uptake (3,4). The adhesion to a program of physical exercise training can improve many metabolic and hormonal functions in different tissues. Muscle and adipose tissue become more responsive to insulin and a reduction in fat depots (both visceral and subcutaneous) has been consistently reported (5,11,13,14,28). The increase in insulin-induced glucose uptake rates reported after chronic exercise is partially due to the rise in the activity and expression of the proteins involved in insulin signaling as well as in the insulin responsive glucose transporter (GLUT4), and this response is particularly known in skeletal muscle (11,17,21,28). However, the effects of endurance exercise training, mainly its influence on the early steps of the insulin signaling pathway in intact adipose tissue or isolated adipocytes, have not been adequately evaluated. In the present work, we analyzed the protein content and the phosphorylation degree of insulin receptor β-subunit (IR-β), insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), and the association of IRS proteins with Phosphatidyl-Inositol-3-kinase (PI3-K). We also determined the ability of insulin to induce serine phosphorylation of AKT/PKB in endurance exercise-trained rats. Our results show that endurance exercise training improved the responsiveness of adipocytes to insulin and that this effect is exerted through the IRS/PI3K/AKT pathway.

MATERIALS AND METHODS

Animals and training schedule. Male Wistar rats (45 days old, weighing 200 g) from the Animal Resources Center of the Institute of Biomedical Sciences of the University of São Paulo were used in the experiments. They were housed in collective cages, with free access to water and food (balanced chow pellet diet, Nuvilab CR1, Nuvital SA, Colombo, PR, Brazil). The animals were randomly distributed into two age-matched groups: sedentary (S) or exercise-trained (T). The last group was submitted to an endurance exercise training program consisting of running sessions on a motor treadmill (KT-300, Inbramed Ltda, Porto Alegre, RS, Brazil), for 1 hour per day, 5 days per week, for 7 weeks. The training schedule was based on Braga et al. (9) with some adaptations. Briefly, the exercise was progressive with increasing intensity, determined by a combination of velocity and duration (0% grade). It began with 5 m/min, 10-15 min/day, reaching 8.3 m/min and 30 min/day at the end of week 1. The training intensity was gradually increased up to 60 min/day (by week 3) with a speed of 16.7 m/min (by weeks 4 to 5) and finally 20 m/min (by weeks 6 to 7). Under these conditions, the training allowed approximately 60% of maximal oxygen consumption (VO_{2max}) for the last two weeks. The protocol used for O_2 consumption testing was performed as described elsewhere (9). The Ethical Committee for Animal Research of the Institute of Biomedical Sciences of the University of Sao Paulo approved all experimental procedures.

Materials. Antiphosphotyrosine, anti-IR-β subunit, anti-IRS-1, and anti-IRS-2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti p85 regulatory subunit of PI3-K antibody was from Upstate Biotechnology, Inc. (Lake Place, NY). Anti-phosphoserine^{473}-AKT antibody was from Cell Signaling Technology, Inc. (Beverly, MA). Reagents for SDS-polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad Laboratories, Inc. (Richmond, CA). Collagenase type II, aprotinin, dithiothreitol, HEPES, phenylmethylsulfonyl fluoride, sodium orthovanadate, Triton X-100, Tween 20, glycerol, D'MEM, bovine serum albumin (BSA, fraction V), and phloretin were from Sigma Chemical Co. (St. Louis, MO). 2-deoxy-D-[^{3}H]-glucose, A_{14}-monoiodo-^{125}I-labeled insulin, protein A-Sepharose 6MB, nitrocellulose membrane (Hybond ECL), ECL™ kit containing secondary horseradish-labeled antibodies, and radiographs (Hyper film®) were from Amersham Biosciences, Inc. (Sao Paulo, SP, Brazil). Human recombinant regular insulin was from Biobrás S.A. (Montes Claros, MG, Brazil). Sodium pentobarbital (Hypnol®) was purchased from Cristalia Prod. Quimicos Ltda (Itapira, SP, Brazil). The glucose kit used for plasma glucose determination was from CELM (Glicose SL-E, Sao Paulo, SP, Brazil), and the kit used for plasma insulin determination was from Linco Research, Inc. (kit # RI-13K, St. Charles, MO). Scintillation cocktail (Eco Lume™) was from ICN Pharmaceuticals (Costa Mesa, CA). Bicinchoninic acid reagents were from Pierce Biotechnology, Inc. (Rockford, IL).

Isolation of adipocytes. The animals were killed (8:00 AM) under pentobarbital anesthesia (4 mg/ 100 g b.w.) by decapitation after a 12 hour fasting and the blood from the trunk was collected. The trained animals were killed 24 h after the last bout of exercise in order to prevent its acute effects. The sedentary rats were also killed at the same moment. Both epididymal fat pads were totally removed, weighed, minced with fine scissors and digested at 37 °C in a medium containing colagenase type II (1.25 mg/ml in /l D'MEM/25 mmol HEPES/4% Bovine serum albumin (BSA), pH 7.4), and the adipocytes were isolated according to Rodbell (31). The isolated adipocytes (approximately 10^5 cell/ml) were suspended in Earle/Hepes/BSA (E.H.B.) buffer, pH= 7.4 at 37 °C. Cell size and number were determined as previously described by Di Girolamo et al. (15).

Determination of the citrate synthase activity in soleus muscle. Citrate synthase maximum activity was determined as previously described by ALP et al. (2). Briefly, under pentobarbital anesthesia (4 mg/ 100 g b.w.), samples (100 mg) of soleus muscle were excised and homogenized twice (10 sec each at maximum speed, Polytron® PT 3100, Kinematica AG, Littau-Lucerne, Switzerland) in 1 ml of extraction buffer (50 mmol/l Tris-HCL and 1 mmol/l EDTA, pH 7.4), and centrifuged (3800 *g*, 30 s, 4 ^{o}C). The supernatant was used to analyze spectrophotometrically (412 nm) the citrate synthase (EC 4.1.3.7) maximum activity. The protein content was quantified using Bicinchoninic acid reagents. The maximal enzyme activity was expressed as nmoles.min^{-1}.mg^{-1} of protein.

Plasma glucose and insulin levels. Plasma glucose was determined by enzymatic glucose-oxidase/peroxidase method (7), with a commercial kit. Insulin levels were determined by radioimmunoassay, using specific antibody against rat insulin. The intra-assay variability was less than 5%.

Insulin-stimulated 2-deoxy-D-[^{3}H]-glucose uptake rates (2DGU). 2DGU experiments were performed as described elsewhere with some modifications (26, 27). Briefly, aliquots (100 μl) of isolated adipocytes (20% cell suspension) were transferred to plastic test tubes (17 × 100 mm) with or without insulin (10 nM) diluted in EHB buffer (pH 7.4, final reaction volume = 400 μl), and the cells were incubated for 30 min in a water bath at 37 °C. At the end of the incubation period, a 40 μl aliquot of the reaction mixture was pipetted in a 2 ml plastic tube containing a 10 μl aliquot of 2-deoxy-D-[^{3}H]-glucose (0.4 mmol/l final concentration and 0.05 μCi/tube), and the uptake reaction was allowed for exactly 3 min. 2DGU was interrupted by adding 250 μl of ice-cold phloretin (0.3 mmol/l in EHB). Next, 200 μl of this last mixture were transferred to microfuge tubes (400 μl capacity) layered with 200 μl of silicone oil (dens. = 0.963 mg/ml), and centrifuged (Microfuge E, Beckman Instruments, Palo Alto, CA) for 9 sec at 15000 rpm. The cell pellet on the top of the oil layer was removed to vials containing 3 ml of scintillation cocktail, and the trapped radioactivity was measured in a liquid scintillation counter (Tri Carb 2100TR, Packard instrument Company, Meriden, CT). Unspecific 2-DG radiolabel trapping was determined in a parallel tube already prepared with 250 μl of ice-cold phloretin to stop transport reaction from the beginning. This value was discounted

from the total trapping, and the resultant specific uptake was recalculated to be expressed as pmol/cm^2 of cell surface area, which is a good index of the glucose transporter population density present in adipocyte plasma membrane.

Insulin binding to cell surface receptors. From the same 20% adipocyte suspension, 450 μl aliquots in EHB (pH 7.8) were transferred to 12 x 75 mm polypropylene test tubes prepared with a 10 μl mixture of A_{14}-monoiodo-^{125}I-labeled insulin (10,000 counts.min^{-1}.tube^{-1}) in the absence or presence of "cold" insulin (0.025, 0.1, 0.25, 1, 2.5 and 10 nM), in a 500 μl final reaction volume. This mixture was incubated for 180 min in a water bath at 16 ^{o}C to prevent receptor internalization (29). The assay was interrupted by the centrifugation of 200 μl aliquots through silicone oil and the radioactivity trapped in the cell pellets on the top of the oil layer was measured as described elsewhere (27). The plasma membrane receptor number was determined according to Scatchard (34).

Tissue extraction, immunoprecipitation and immunoblotting. These analyses were performed as described elsewhere (32,36). Briefly, rats, under pentobarbital anesthesia (4 mg/100 g b.w.), received an intravenous "bolus" injection of regular insulin (50 nmol/l/100 g b.w) through the abdominal vena cava. Immediately before the injection and 90 sec after it [at this time point, the maximal insulin-induced phosphorylation response was obtained, as determined by a time-course study performed in our laboratory], the peri-epididymal fat pads were excised and processed for analysis of IR-β, IRS-1, IRS-2 and AKT [in this case we observed

two peaks of insulin-induced AKT phosphorylation: at 90 sec and at 5 min] studies. The fat was immediately homogenized in freshly prepared ice-cold buffer (1% Triton X-100, 100 mmol/l sodium orthovanadate, EDTA 10 mmol/l, TRIS 100 mmol/l, sodium pyrophosphate 10 mmol/l, sodium fluoride 100 mmol/l, 2 mmol/l phenyl-methyl-sulphonyl fluoride and 0.01 mg/ml aprotinin, pH=7.5) using a Polytron® homogenizer (PT 3100) set at maximum speed for 30 sec. Insoluble material was removed by centrifugation (15000 x *g*) for 20 min at 4 °C. Protein concentration was estimated by Biuret method (16). Aliquots of the resulting supernatants containing 3 mg of total protein were immunoprecipitated with anti-IR-β subunit or anti-IRS-1 or anti-IRS-2 antibodies. The antibodies were added to homogenates and incubated overnight at 4 °C, followed by the addition of protein A sepharose 6MB for 2 hours. The mixture was centrifuged (15000 x *g*, for 15 min at 4 ^{o}C) and the pellets were washed three times in ice-cold buffer (0.5% Triton X-100, 100 mmol/l Tris, pH 7.4 10 mmol/l EDTA and 2 mmol/l sodium ortovanadate), and then resuspended in Laemmli sample buffer (0.1% bromophenol blue, 1 mmol/l sodium phosphate (pH=7.0), 50% glycerol and 10% sodium dodecyl sulfate), boiled for 5 min before SDS-PAGE (6.5% bis-acrylamide) in a miniature slab gel (Mini-Protean, Bio-Rad Laboratories, Inc., Richmond, CA). Proteins in gel were electrophoretically transferred for 50 min (120 V) at 4 ^{o}C to a nitrocellulose membrane. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the membrane in blocking buffer (5% (w/v) nonfat dry milk, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween-20) for 4 h at 4 ^{o}C. The membranes were blotted with anti-phosphotyrosine (1μg/ml, 1:400), anti-IR-β subunit (1:400), anti-

IRS-1 (1:400), or anti-IRS-2 (1:400) antibodies diluted in blocking buffer (3% BSA instead of nonfat dry milk). To check if PI3-K co-immunoprecipitated with IRS-1 and IRS-2, the membranes were washed (90 min) three times for stripping the first antibody using a buffer (pH=2.8) containing 200 mmol/l glycine, 200 mmol/l NaCl, and NaOH 1N and re-blotted with an antibody against the anti-p85 regulatory subunit of PI3-K (1:5000). For the determination of the phosphorylation of AKT, total extracts of peri-epididymal fat pads were prepared and 75 μg total protein were resolved by SDS-PAGE (8% bis-acrylamide), transferred to nitrocellulose membranes and blotted with anti-phosphoserine (Ser^{473}) AKT (1:1000) antibody. Each membrane was subsequently incubated with a secondary horseradish-labeled antibody for 1 hour followed by the addition of substrate reaction mix for a chemiluminescent detection of the protein specimen. Results were visualized using Amersham Hyper Film®. Band intensities of the exposed X-ray films were quantified by optical densitometry (Scion Image® Software 4.02 – Scion Corporation, Frederick, MD). Results were normalized assuming the mean of densitometric measurements of bands from the fat tissue of sedentary animals before insulin stimulation as 1 arbitrary unit (AU).

Data presentation and statistical analysis. All data are expressed as mean ± SEM. Student's *t* test for unpaired samples was used to test for differences between the groups for all parameters. For insulin signaling studies, two-way ANOVA for repeated measures and Bonferroni post hoc tests were adopted. A *P* value of 0.05 was established as a fiducial limit of significance.

RESULTS

Body weight, body weight gain, epididymal fat pad weight and adipocyte volume. As shown in table 1, final body weight, body weight gain (Δ, final body weight - initial body weight) and epididymal fat pad weight from both groups were not statistically different. However, adipocyte volume from trained rats decreased after 7 weeks of training when compared to sedentary rats.

Citrate synthase activity in soleus muscle. Citrate synthase activity in soleus muscle was taken as an index of training effectiveness (25). As table 1 shows, citrate synthase activity increased 40% ($p<0.05$) in trained rats when compared to sedentary rats.

Plasma glucose and insulin levels. Plasma glucose and insulin are presented in Table1. Trained rats exhibited a 7% decrease ($p<0.05$) in plasma glucose and a 42% reduction ($p<0.05$) in insulin levels in comparison to sedentary rats.

Insulin-stimulated 2-deoxy-D-[^{3}H-glucose uptake rates (2DGU). The uptake of the non-metabolizable glucose analogue 2-deoxy-D-[^{3}H]-glucose was measured in isolated fat cells from trained and sedentary rats, both in the absence or in the presence of 10 nM insulin. This modified hexose, like glucose, is transported through the cell membrane by the same saturable transport system and is phosphorylated by hexokinase but not further metabolized, accumulating as 2-deoxy-D-glucose-6-phosphate (30). As shown in Table1, adipocytes from trained

rats increased insulin-induced glucose uptake (90 %, $p<0.05$) when compared to the sedentary rats. Basal rates of glucose uptake were not affected by training.

Insulin binding to cell surface. Table 1 shows the number of insulin receptors in fat cells surfaces estimated by Scatchard analysis (34). The receptor number did not show any statistical difference between the groups, indicating that the differences in 2DGU are probably influenced by post-receptor mechanisms.

Insulin receptor β-subunit. Peri-epididymal adipose tissues were excised before and after an *i.v.* insulin injection and subsequently homogenized in ice-cold extraction buffer (4^{o} C). Western blot analysis of the immunoprecipitated IR-β subunit was performed to quantify protein content and the degree of tyrosine phosphorylation of IR-β (PY-IR-β) *(to further details see material and methods)*. These parameters did not differ significantly between the groups, neither before nor after insulin stimulation.

IRS-1 and PI3-K association. Fig. 2*A* shows a representative blot of the immunoprecipitated IRS-1 protein content. There were no significant differences between the groups. Insulin-induced tyrosine phosphorylation of IRS-1 (PY-IRS-1) increased 57% ($p<0.05$) in trained rats when compared to sedentary rats (Fig. 2*B*). The following step in insulin signaling, the PY-IRS-1 association to the regulatory p85 subunit of PI3-K, was also determined (Fig. 2*C*). After insulin injection, the

adipose tissue from the trained rats showed a 1.57 fold increase ($p<0.05$) in IRS-1/PI3-K association when compared to sedentary rats.

IRS-2 and PI3-K association. Fig. 3*A* shows a representative blot of the immunoprecipitated IRS-2 protein content. There were no significant differences between the groups. Our results show that trained rats increased basal (1.4 fold, $p<0.05$) and insulin-stimulated tyrosine phosphorylation of IRS-2 (PY-IRS-2) (2.38 fold, $p<0.05$) when compared to sedentary rats (Fig. 3*B*). However, the insulin-induced IRS-2/PI3-K association was not significantly different in both groups (Fig. 3*C*).

AKT (Fig. 4). To determine whether PI3-kinase downstream signaling was affected by training, we measured AKT serine^{473} phosphorylation (P-S^{473}-AKT) in peri-epididymal fat pads excised before and 90 and 300 sec after insulin injection. After 90 sec, the adipose tissue from trained rats showed a 3.15-fold increase ($p<0.05$) in P-S^{473}-AKT when compared to the sedentary rats. However, after 5 min there were no statistical differences between the groups.

DISCUSSION

Endurance exercise training has been reported to increase insulin action in experimental models using animals or humans. This adaptation is particularly prevalent in muscle and white adipose tissue. At the adipocyte level, the improved insulin response is accompanied by a reduction in mean cell diameter after long lasting exercise sessions in humans and animals (12, 13, 14). Endurance exercise trained rats exhibited increased 2DGU in comparison to sedentary age-matched rats after insulin stimulation. Our findings are in accordance with previous reports indicating that chronic exercise induces an increase in insulin responsiveness not only in muscles but also in adipocytes that exhibited a reduction in size (11, 13, 14, 17, 39). Other researchers attributed this adaptation to an augment in insulin receptor numbers, which was not observed in the present work. However, the training schedules adopted in those studies were considerably different since the animals were submitted to prolonged swimming exercise sessions (6 h/day) (14, 37). Further, the augment (18%) in insulin binding was not enough to explain the intense increase (approximately 4 times) in adipocyte insulin responsiveness (37). In conclusion, our result is in accordance with a previous study where rats trained by treadmill running did not show any differences in insulin binding (38).

Intracellular events linking insulin binding to its receptor and the glucose uptake by GLUT4 translocation to plasma membrane have been studied in models of chronic and acute exercise and used to explain how insulin controls its biological effect (19, 21, 28). Here we analyzed the early steps of insulin signaling in peri-epididymal fat pads from sedentary and trained rats in an attempt to elucidate the increased insulin responsiveness observed after exercise training. The first step of

insulin signaling transduction pathway is the auto-phosphorylation of IR-β subunit after insulin binding to its extracellular α-subunit. Such phenomenon triggers tyrosine kinase activation of IR-β subunit that phosphorylates a wide range of proteins in the cytosol and on the plasma membrane inner surface (33). In our study, we did not observe any differences in PY-IR-β and its protein content after 7 weeks of physical training in rats. On the other hand, studies conducted in skeletal muscle homogenates have demonstrated controversial results about the effects of endurance exercise training on the first step of insulin signaling. For instance, Chibalin et al. (10) reported an increased IR-β protein content and phosphorylation in skeletal muscle after 5 days of training in rats. However, Yu et al. (40) showed a reduction in protein content of IR-β in vastus lateralis from endurance exercise runners. In our previous work we did not observe difference in PY-IR-β or protein content in rats after 6 weeks of swimming training (28). Possibly, different schedules of training, and species-specific or tissue-specific factors might justify some discrepancies found in these studies, indicating that further work will be needed to clarify this issue.

As described above, regular exercise did not change the protein content of IRS-1 and IRS-2 after endurance exercise training, although several studies have demonstrated discrepancies, like reductions in IRS-1 and -2 (40), no differences (28), or augmented IRS-2 expression (10). We also studied the insulin-induced PY-IRS-1 and PY-IRS-2. Apart from protein content, chronic exercise was efficient to augment the phosphorylation of both IRS isoforms when compared to sedentary animals after insulin stimulation. IRS-1 and -2 are key proteins in insulin responsive

cells since they link the membrane-generated signal to PI3-K, activating this enzyme which will ultimately recruit downstream proteins involved in the GLUT4 translocation from *trans*-Golgi to plasma membrane and subsequently increasing glucose transport (22,24). In our study, an increased insulin-stimulated IRS-1/PI3-K association was found in the trained group. Interestingly, the amount of association showed almost a stoichiometric correlation with glucose transport and PY-IRS-1. Notwithstanding basal and insulin-stimulated PY-IRS-2 were increased in adipose tissue homogenates from trained rats, we observed only a slight tendency to increase the IRS-2/PI3-K association. IRS-2 may likely be associated to other docking proteins as Grb2, which in conjunction with Shc, SOS and Ras, operates as a molecular switch stimulating the MAP kinase pathway and subsequent gene expression (33), but this was not explored here. In addition, it is important to note that only the association of IRS-1, -2 to PI3-K, does not reflect the activity of the enzyme and consequently, IRS-2 must not be completely ruled out. Hence, the increased insulin responsiveness of adipocytes from endurance exercise-trained rats seems to be mediated preferentially by IRS-1 regardless of reports showing that adipocytes from IRS-2 *knockout* mice elicited a diminished glucose uptake and the overexpression of IRS-1 did not fully reestablish this insulin-mediated effect (6, 18). However, these two studies were not performed in exercised or trained mice. In another work with IRS-2 *knockout* mice subjected to an acute exercise, an increase in muscle glucose uptake was observed (20).

To examine more distal steps in insulin signaling through the PI3-K pathway, we determined the capacity of insulin to induce serine AKT phosphorylation (P-S^{473}-AKT), a protein with a pleckstrin domain and pleiotropic actions. This

downstream insulin effector is directly activated by a product of PI3-K, phosphotidylinositol-3-phosphate and additionally modulated by its upstream activator, phosphoinositide-dependent kinases 1 and 2 (PDK-1, 2), event considered an essential step in glucose uptake (1). We found increased P-S^{473}-AKT after 90 sec of insulin stimulation in trained rats when compared to sedentary rats, but not after 5 min (300 sec). As previously demonstrated, gastrocnemius from trained rats showed an increased P-S^{473}-AKT after 5 min of insulin stimulation (28). In this work, AKT content in muscle did not change with training. Therefore, as our results show similar effects in white adipose tissue, we propose that AKT phosphorylation represents an important event to explain the increased insulin-induced glucose uptake of endurance-exercised trained rats.

The data observed in adipose tissue from trained rats are in agreement with our previous report conducted in striated skeletal muscle showing that proteins involved in the first steps of insulin signal transduction mediate the increased insulin responsiveness (28). At least in muscles, PI3-K/AKT pathway is an important contributor to the improvement in insulin-induced glucose transport after training. Although the same pathway seemed to be important in the adipose tissue, alternative routes probably might be implicated. Recent data have proposed the involvement of atypical PKC λ/ζ, and the lipid raft complex as strong mediators (6,35). It is important to note that this is the first experimental observation showing the effects of endurance exercise training through the first steps of insulin signaling in adipocytes from trained rats.

In summary, the results of our study show that: 1) endurance exercise training improved insulin responsiveness in isolated adipocytes; 2) this adaptation is not a consequence of the increased insulin binding to adipocytes, and 3) this improvement in glucose uptake involves at least the IRS/PI3-K/AKT pathway, and it seems that IRS-1 (more than IRS-2) is preferentially implicated.

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TABLE 1. General characterisitics of sedentary and trained rats.

	S	T
Body weight, *g* (20)	297 ± 4.8	290 ± 7.2
Body weight gain (7 weeks), *g* (20)	104 ± 4.4	107 ± 2.1
Epididymal fat pad, *g* (20)	1.41 ± 0.05	1.36 ± 0.06
Adipocyte volume, *pl* (10)	286 ± 26.4	205 ± 16.8 *
Citrate synthase activity, *nmol.min^{-1}.mg^{-1} protein* (10)	364.5 ± 16.6	512.4 ± 31.0 *
Plasma glucose, *mmol/l* (10)	5.3 ± 0.07	4.9 ± 0.13 *
Insulin levels, *nmol/l* (10)	0.41 ± 0.049	0.24 ± 0.012 *
2DGU, *pmol^{1}.cm^{-2}* (10)		
basal	6.5 ± 1.6	7.3 ± 2.4
insulin (10 nmol/l)	12.1 ± 2.9	23.1 ± 3.1 *
Insulin receptors, *x 10^{3}* (10)	145 ± 15	167 ± 12

Data are means ± SEM. *p<0.05 vs. S;

The number in parenthesis represents the *n* for each parameter.

Legends

Fig. 1. IR-β protein content (A) and IR-β tyrosine phosphorylation (B) in epididymal fat pads from trained and sedentary age-matched rats. Scanning densitometry was performed on autoradiographs from 6 experiments. Data are expressed as mean ± SEM. Results were normalized assuming the mean of densitometric measurements of bands from the fat tissue of sedentary animals before insulin stimulation as 1 arbitrary unit (AU). The bars are aligned with the respective blots in the upper part of figure.

Fig. 2. IRS-1 protein content *(A)*, IRS-1 tyrosine phosphorylation (*B)* and IRS-1/PI3-kinase (p85 subunit) association (*C)* in epididymal fat pads from trained and sedentary age-matched rats. Scanning densitometry was performed on autoradiographs from 6 experiments. Data are expressed as mean ± SEM. Results were normalized assuming the mean of densitometric measurements of bands from the fat tissue of sedentary animals before insulin stimulation as 1 arbitrary unit (AU). The bars are aligned with the respective blots in the upper part of figure. *$p<0.05$.

Fig. 3. IRS-2 protein content (*A)*, IRS-2 tyrosine phosphorylation (*B)* and IRS-2/PI3-kinase (p85 subunit) association (*C)* in epididymal fat pads from trained and sedentary age-matched rats. Scanning densitometry was performed on

autoradiographs from 6 experiments. Data are expressed as mean ± SEM. Results were normalized assuming the mean of densitometric measurements of bands from the fat tissue of sedentary animals before insulin stimulation as 1 arbitrary unit (AU). The bars are aligned with the respective blots in the upper part of figure. *p<0.05.

Fig. 4. Insulin-stimulated AKT serine^{473} phosphorylation in epididymal fat pad from trained and sedentary age-matched rats. Scanning densitometry was performed on autoradiographs from 6 experiments. Data are expressed as mean ± SEM. Results were normalized assuming the mean of densitometric measurements of bands from the fat tissue of sedentary animals before insulin stimulation as 1 arbitrary unit (AU). The bars are aligned with the respective blots in the upper part of figure. *p<0.05.

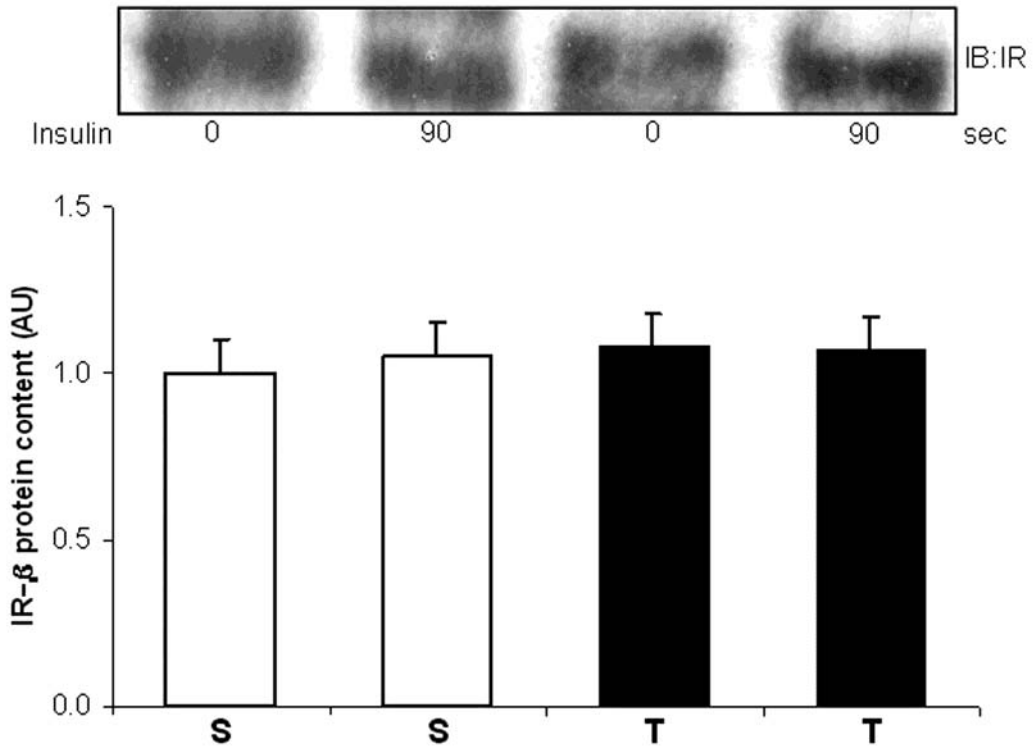
IB:IR
Insulin
0
90
0
90
sec
1.5
1.0
0.5
0.0
IR-β protein content (AU)
S
S
T
T

Figure 1A

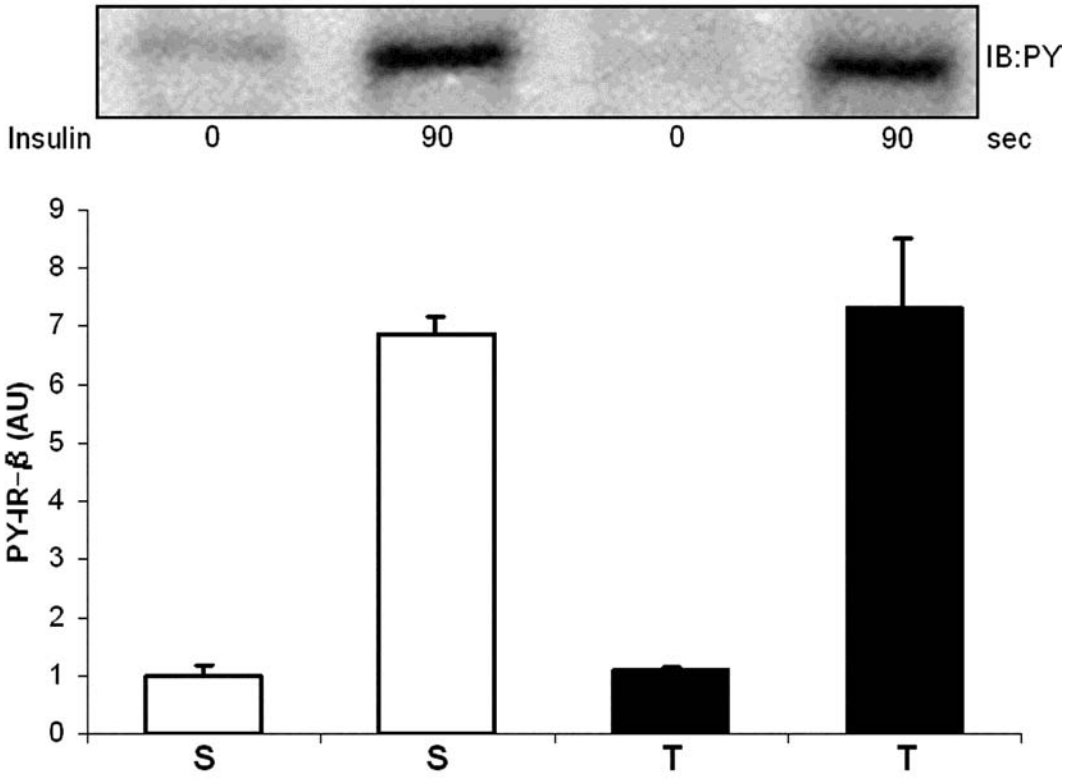

Figure 1B

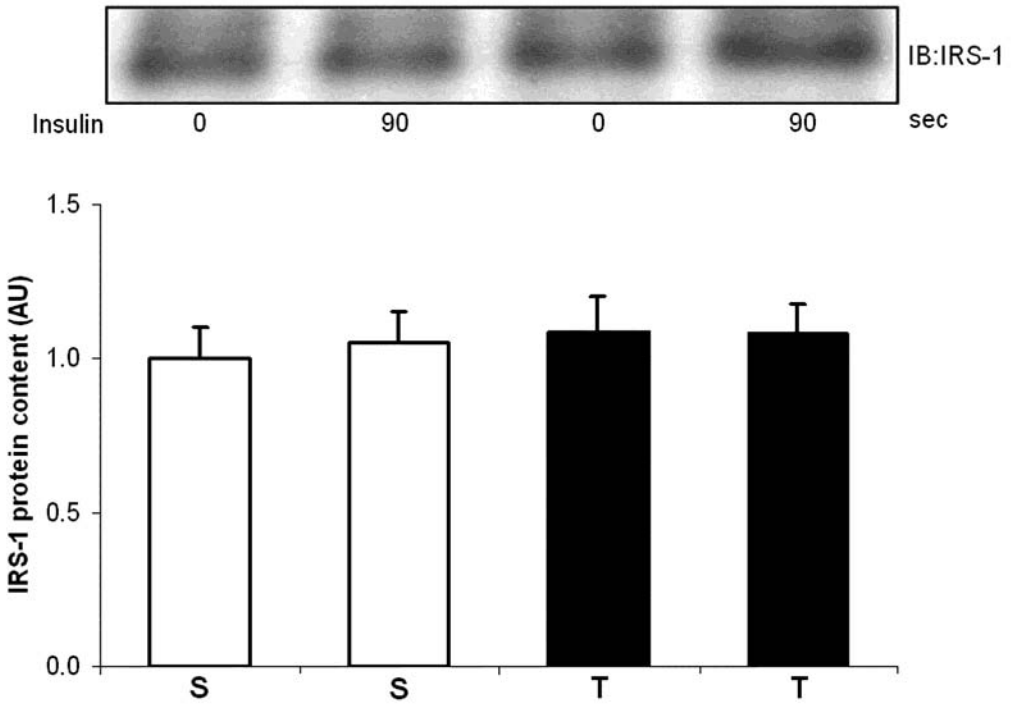
IB:IRS-1
Insulin
0
90
0
90
sec
IRS-1 protein content (AU)
1.5
1.0
0.5
0.0
S
S
T
T

Figure 2A

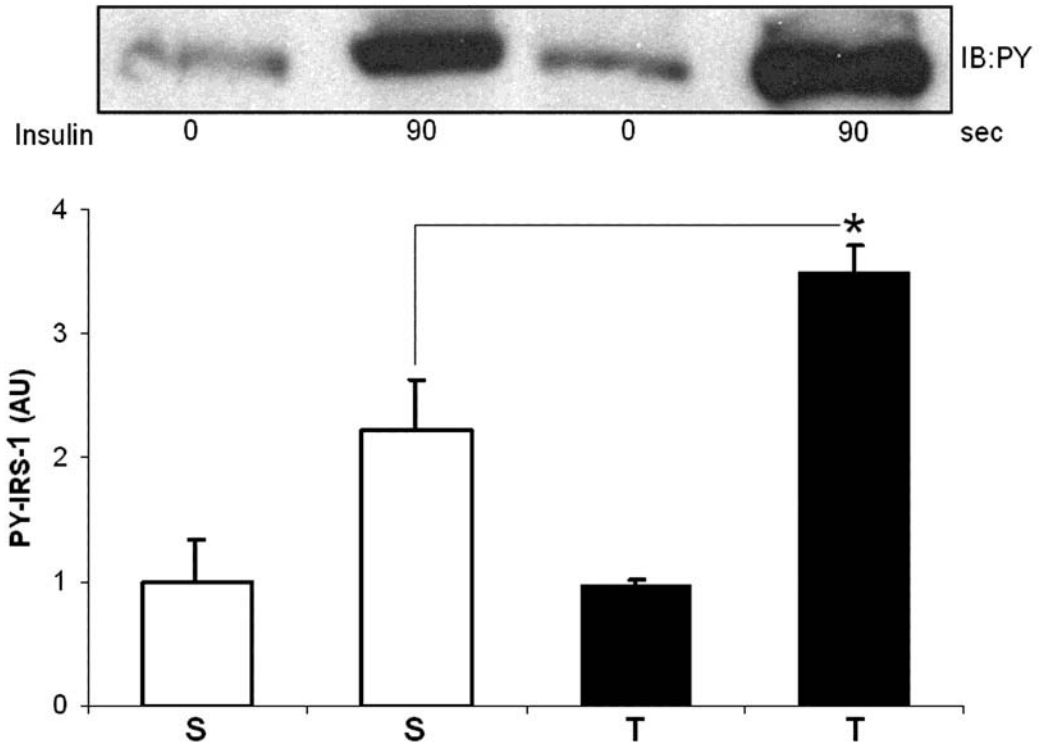

Figure 2B

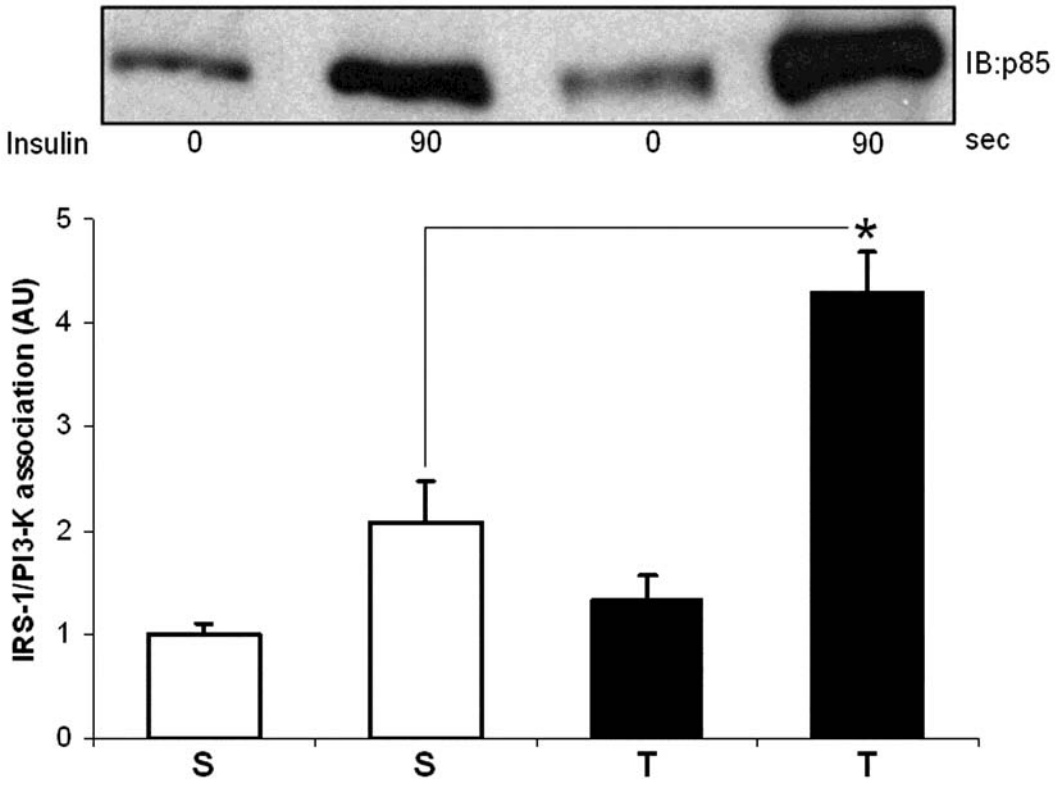
IB:p85
Insulin
0
90
0
90
sec
IRS-1/PI3-K association (AU)
5
4
3
2
1
0
*
S
S
T
T

Figure 2C

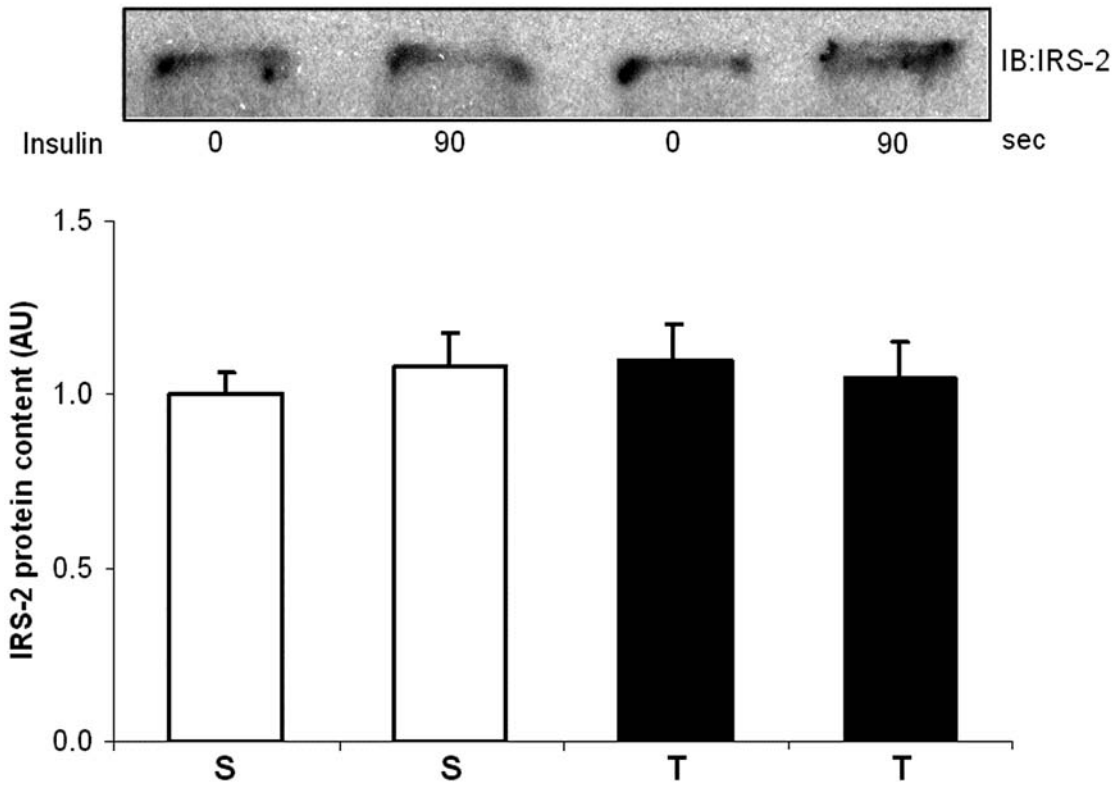
IB:IRS-2
Insulin
0
90
0
90
sec
IRS-2 protein content (AU)
1.5
1.0
0.5
0.0
S
S
T
T

Figure 3A

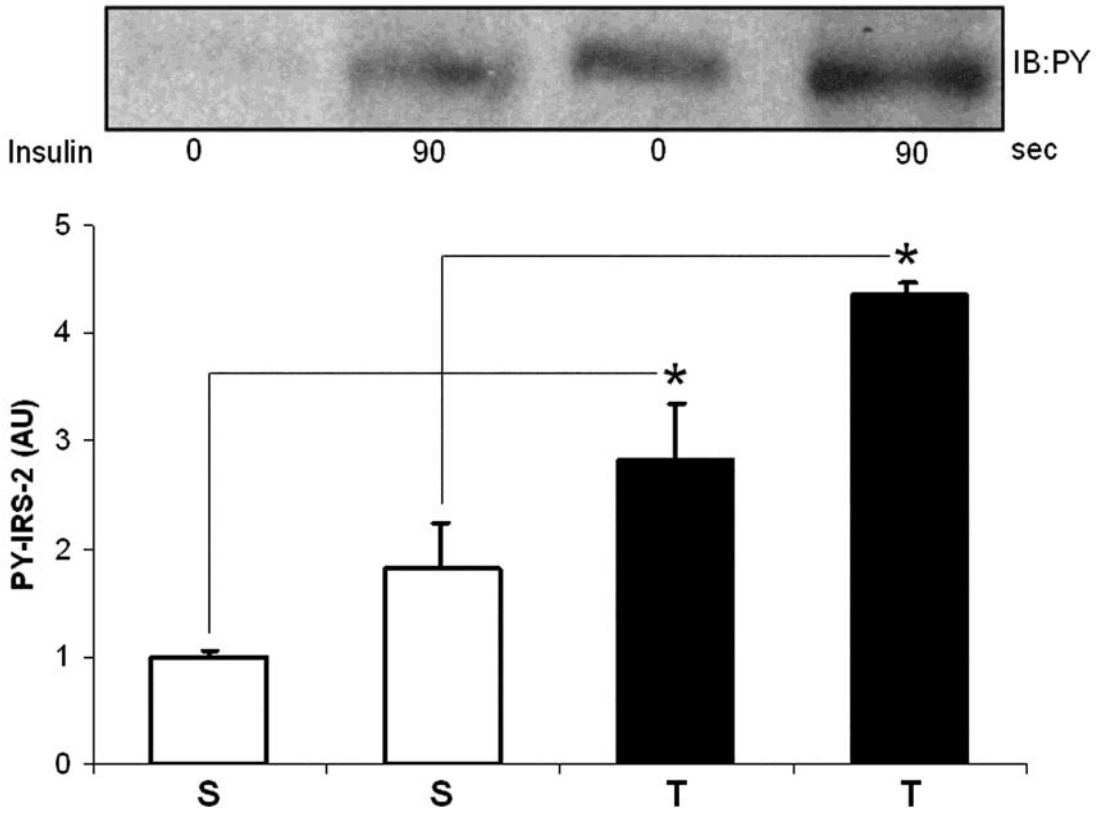

Figure 3B

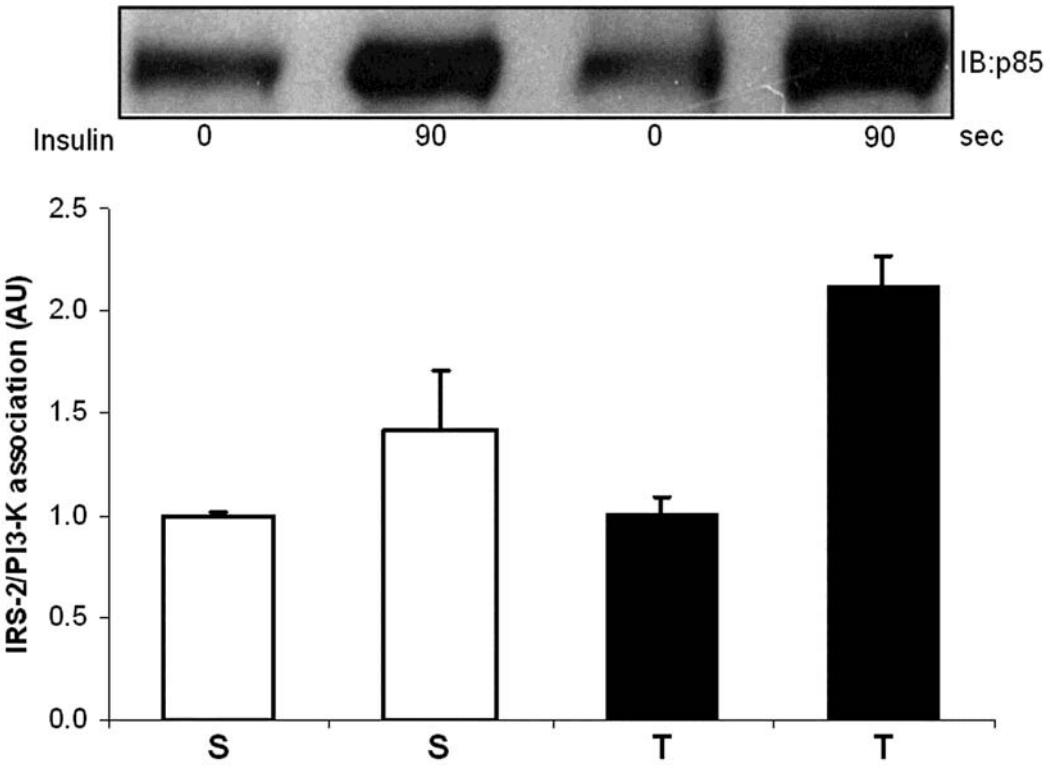
IB:p85
Insulin
0
90
0
90
sec
IRS-2/PI3-K association (AU)
2.5
2.0
1.5
1.0
0.5
0.0
S
S
T
T

Figure 3C

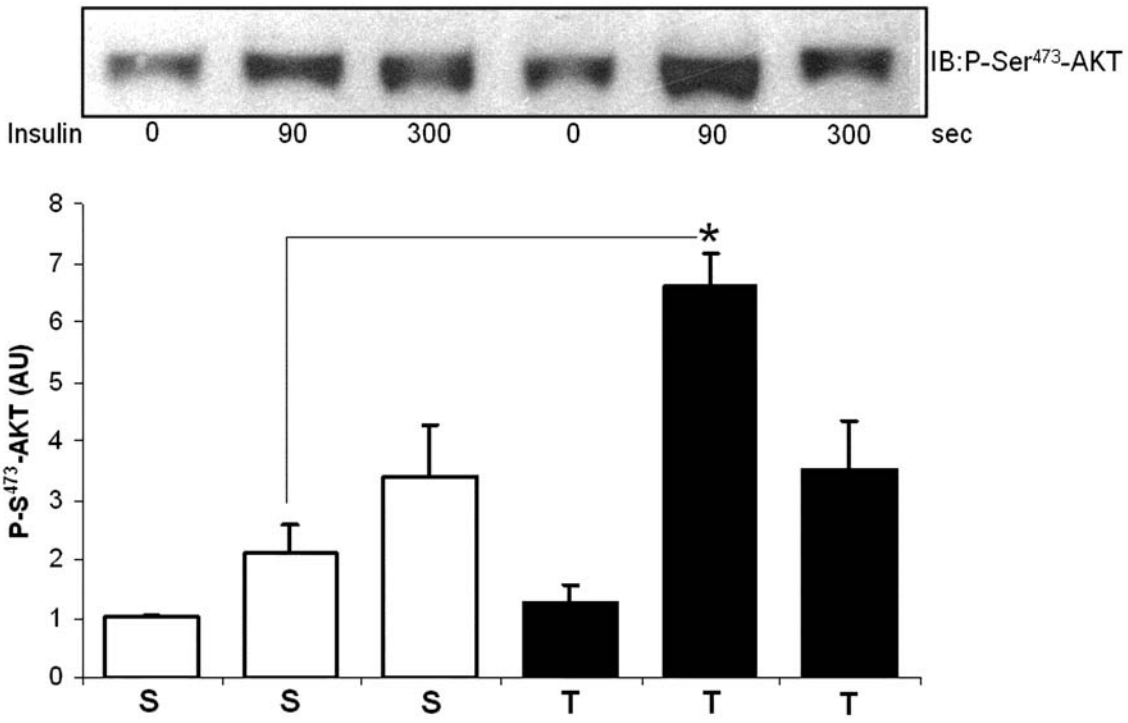
IB:P-Ser473-AKT
Insulin
0
90
300
0
90
300
sec
P-S473-AKT (AU)
0
1
2
3
4
5
6
7
8
*
S
S
S
T
T
T

Figure 4