

EXPERIMENTAL STUDY

Endurance training improves responsiveness to insulin and modulates insulin signal transduction through the phosphatidylinositol 3-kinase/Akt-1 pathway

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Abstract

Background: Endurance training increases insulin-stimulated muscle glucose transport and leads to improved metabolic control in diabetic patients.

Objective: To analyze the effects of endurance training on the early steps of insulin action in muscle of rats.

Design: Male rats submitted to daily swimming for 6 weeks were compared with sedentary controls. At the end of the training period, anesthetized animals received an intravenous (i.v.) injection of insulin and had a fragment of their gastrocnemius muscle excised for the experiments.

Methods: Associations between insulin receptor, insulin receptor substrates (IRS)-1 and -2 and phosphatidylinositol 3-kinase (PI3-kinase) were analyzed by immunoprecipitation and immunoblotting. Akt-1 serine phosphorylation and specific protein quantification were detected by immunoblotting of total extracts, and IRS-1/IRS-2-associated PI3-kinase activity were determined by thin-layer chromatography.

Results: Insulin-induced phosphorylation of IRS-1 and IRS-2 increased respectively by 1.8-fold ($P < 0.05$) and 1.5-fold ($P < 0.05$), whereas their association with PI3-kinase increased by 2.3-fold ($P < 0.05$) and 1.9-fold ($P < 0.05$) in trained rats as compared with sedentary controls, respectively. The activity of PI3-kinase associated with IRS-1 and IRS-2 increased by 1.8-fold ($P < 0.05$) and 1.7-fold ($P < 0.05$) respectively, in trained rats as compared with their untrained counterparts. Serine phosphorylation of Akt-1/PKB increased 1.7-fold ($P < 0.05$) in trained rats in response to insulin. These findings were accompanied by increased responsiveness to insulin as demonstrated by a reduced area under the curve for insulin during an i.v. glucose tolerance test, by increased glucose disappearance rate during an insulin tolerance test, and by increased expression of glucose transporter-4.

Conclusions: The increased responsiveness to insulin induced by chronic exercise in rat skeletal muscle may result, at least in part, from the modulation of the insulin signaling pathway at different molecular levels.

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Introduction

Regular exercise improves metabolic control in diabetic individuals and is an important component of treatment in diabetes mellitus (1). In humans and experimental animals, endurance training improves insulin-induced glucose uptake (2, 3) and reduces glucose-stimulated insulin secretion (4), thereby contributing to glucose homeostasis. Hyperinsulinemic–euglycemic clamp studies have shown a greater insulin-stimulated glucose uptake in both acutely and endurance-trained individuals (5). However, in untrained diabetic individuals, a

single bout of exercise may precipitate ketosis and consequently decrease glucose uptake (6). Several mechanisms may act locally to improve glucose uptake and disposal after exercise. These include increased muscle blood flow, increased insulin binding to its receptor (the insulin receptor, IR) and increased IR turnover (3, 5, 7, 8). The principal events that link insulin to glucose uptake include insulin binding to its receptor, leading to IR autophosphorylation and triggering of IR kinase activity towards insulin receptor substrates (IRS)-1 and -2. Once phosphorylated, IRS-1 and -2 bind to and activate phosphatidylinositol 3-kinase (PI3-kinase), an

essential element in the regulation of glucose uptake (9–13). Different approaches have demonstrated that Akt-1/PKB, a serine-threonine kinase with a pleckstrin homology domain, is a downstream effector of PI3-kinase and one of the potential mediators of the insulin signals for glucose uptake (14, 15).

Recent studies have analyzed the effects of short-term training upon elements of the insulin signaling pathway. Chibalin and coworkers (16) found an increased phosphorylation of IRS-1 and -2 and an increased associated PI3-kinase activity after 5 days of training in Wistar rats. Wojtaszewski and coworkers (17), found increased glucose uptake and glycogen synthase activity after 1 h of one-leg exercise in young healthy humans, with no significant change in proximal signaling events of the insulin signaling pathway.

In the present work, we analyzed the protein concentrations and phosphorylation status of IR, IRS-1 and IRS-2, and the association of IRS-1/-2 with PI3-kinase, in addition to insulin-induced PI3-kinase activation and Akt-1/PKB serine phosphorylation, in sedentary and endurance-trained Wistar rats, after stimulation with insulin *in vivo*.

Materials and methods

Antibodies and chemicals

Monoclonal anti-phosphotyrosine antibody and polyclonal antisera against glucose transporter (GLUT)-4, IR, IRS-1, IRS-2, and PI3-kinase p85-subunit were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal antiserum against phosphoserine (Ser 473) Akt-1 was from Upstate Biotechnology (Lake Placid, NY, USA). [¹²⁵I]Protein A, [¹²⁵I]insulin and nitrocellulose membranes were from Amersham (Amersham, Bucks, UK). Protein A Sepharose 6MB was from Pharmacia (Uppsala, Sweden). Chemicals were from Sigma (St Louis, MO, USA). Sodium amobarbital and insulin were from Eli Lilly (Indianapolis, IN, USA).

Animals

Male Wistar rats (48 days old/150–200 g) from the University of Campinas Central Animal Breeding Center were used in the experiments. The animals were divided into two groups: sedentary (also referred to as control) and trained. The control rats were kept sedentary throughout the experimental period and the trained rats were submitted to 1 h of swimming daily, in water at 32 °C, with an attached weight corresponding to 5% of body weight. Training occurred 5 days/week for 6 weeks. The rats were allowed free access to standard rodent chow and water, and food was withdrawn 12 h before the experiments, with no acute bout of exercise preceding tissue collection. The rats were anesthetized with intraperitoneal sodium

amobarbital (15 mg/kg body weight), and were submitted to the experimental procedure after the loss of corneal and pedal reflexes.

Hormone and biochemical measurements

Glucose was measured by the glucose oxidase method in samples collected from the tail. Glycogen in fragments of gastrocnemius muscle and liver was detected as previously described (18). Insulin was detected by RIA, utilizing a guinea pig anti-rat insulin antibody and rat insulin as standard (19).

Intravenous glucose tolerance test (GTT) and estimation of insulin action *in vivo* using the 15-min insulin tolerance test (ITT)

An intravenous (i.v.) GTT was performed at the end of the 5th week of training, 48 h after the last training session. Food was withdrawn and the rats were anesthetized as described above. After the collection of an unchallenged sample, a solution of 20% glucose (2 g/kg body weight) was injected through the penile vein, and further samples were collected at 3, 5, 10, 15 and 30 min, for determination of glucose and insulin concentrations. The 15-min ITT was performed 48 h after the last training session. Food was withdrawn 12 h before the test, 6 µg insulin was injected through the penile vein and blood samples were collected at 0, 4, 8, 12 and 16 min. The rate constant for glucose disappearance (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$. The glucose $t_{1/2}$ was calculated from the slope of the least-square analysis of the plasma glucose concentration during the linear decay phase (20).

Tissue extraction, immunoblotting and immunoprecipitation

Tissue extraction, immunoblotting and immunoprecipitation were performed as described elsewhere (21–23). Briefly, rats received an infusion of insulin (6 µg insulin, which produces a blood insulin concentration of about 10^{-6} mol/l) (21) or saline alone (0.5 ml) injected via the portal vein. Whole gastrocnemius muscle was excised 90 s later [the time point of maximal insulin-induced response as determined by time-course (data not shown) and in previous publications (22)], for analysis of GLUT-4, IR, IRS-1 and IRS-2, and 5 min later for PI3-kinase and Akt studies. It was immediately homogenized in freshly prepared ice-cold buffer (1% Triton X-100, 100 mmol/l Tris, pH 7.4, 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium vanadate, 2 mmol/l phenyl methylsulphonyl fluoride and 0.01 mg/ml aprotinin). Insoluble material was removed by centrifugation (50 000 g) for 25 min at 4 °C. Aliquots of the resulting supernatants containing 3 mg

of total protein were used for immunoprecipitation with anti-insulin receptor, anti-IRS-1 or anti-IRS-2 antibodies at 4°C overnight, followed by the addition of Protein A Sepharose 6MB for 1 h. 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 vanadate), and then resuspended in Laemmli sample buffer, and boiled for 5 min before SDS-PAGE (6.5% bis-acrylamide) in a miniature slab gel apparatus (Bio Rad, Richmond, CA, USA). Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant). The nitrocellulose transfers were probed with anti-phosphotyrosine (1 µg/ml), anti-insulin receptor (1:1000), anti-IRS-1 (1:1000), anti-IRS-2 (1:1000) or anti-PI3-kinase p85 (1:5000). For GLUT-4 and anti-phosphoserine (Ser 473) Akt-1 detection, total extracts of gastrocnemius muscle were prepared and 0.2 mg total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-GLUT-4 (1:1000) or anti-phosphoserine (Ser 473) Akt-1 (1:2000) antibodies. The blots were subsequently incubated with [¹²⁵I] protein A. Results were visualized by autoradiography using preflashed Kodak XAR film. Band intensities were quantified by optical densitometry of developed autoradiographs (ImageQuant software).

Determination of PI3-kinase activity in samples from muscle immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies

PI3-kinase activity was measured as described previously (23). Briefly, immunoprecipitates collected with protein A Sepharose 6MB were washed three times in ice-cold PBS, pH 7.4, containing 1% Triton X-100 and 100 µmol/l Na₃VO₄, three times in ice-cold 100 mmol/l Tris (pH 7.5) containing 100 mmol/l LiCl₂ and 100 µmol/l Na₃VO₄, and finally, twice in ice-cold 10 mmol/l Tris (pH 7.5), containing 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 µmol/l Na₃VO₄. The immunocomplexes were resuspended in 50 µl ice-cold 10 mmol/l Tris (pH 7.5) containing 100 mmol/l NaCl, 1 mmol/l EDTA, 10 µl 100 mol/l MgCl₂, and 10 µl phosphatidylinositol (2 µg/µl) sonicated in 10 mmol/l Tris (pH 7.5) and 1 mmol/l EDTA. The reaction was started by the addition of 10 µl 440 µmol/l ATP containing [³²P]ATP and lasted 10 min at 22°C under continuous vortex. To stop the reaction, 20 µl 8 mol/l HCl and 160 µl methanol-chloroform (1:1) were added, and after short centrifugation, the lower phase was applied to silica thin-layer chromatography (TLC) plates coated with 1% (wt/vol) potassium oxalate. TLC was run in CHCl₃-CH₃OH-H₂O-NH₄OH (60:47:11.3:2), and spots were visualized by autoradiography and quantified by densitometry.

Data presentation and statistical analysis

All numerical results are expressed as the mean ± s.d. of the indicated number of experiments. The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by densitometry using ImageQuant software. The graphic representations depict the values obtained for insulin-stimulated controls (sedentary) as 100% (fold-stimulation = 1.0); the remaining conditions are depicted as variations of that value. The Tukey-Kramer test for multiple comparisons was used for analysis. The level of significance was set at $P < 0.05$.

Results

There were no differences in the final body weight, total food intake, and basal glucose concentrations between the sedentary and trained groups at the end of the 6 weeks of training. Resting hepatic and muscle glycogen stores were increased in trained rats (6.22 ± 0.74 mg/100 g tissue compared with 7.67 ± 1.03 mg/100 g tissue, $P < 0.05$, for liver, and 0.48 ± 0.05 mg/100 g tissue compared with 0.59 ± 0.09 mg/100 g tissue, $P < 0.05$, for muscle, in the sedentary and trained groups respectively). The fasting glucose concentrations were similar in both groups, but there was a significant difference between trained and sedentary rats in the plasma glucose responses during the initial minutes of the i.v. GTT (Fig. 1A). However, the total area under the curve for glucose was similar in sedentary and trained rats (Fig. 1A, inset). The insulin concentrations during the i.v. GTT were different between the groups (Fig. 1B), with a marked reduction in the area under the curve in the trained rats compared with that in sedentary animals (0.30 ± 0.02 pg/ml/min compared with 0.18 ± 0.01 pg/ml per min, $P < 0.05$, for sedentary and trained animals respectively; Fig. 1B, inset). After endurance training, there was an increase in responsiveness to insulin as reflected by the glucose disappearance rates (K_{ITT}) of $3.54 \pm 0.35\%$ /min for sedentary rats and $4.25 \pm 0.31\%$ /min for trained rats ($P < 0.05$; Fig. 1C). Finally, the expression of the glucose transporter GLUT-4, final mediator of glucose entrance in skeletal muscle, was increased by 1.7-fold ($P < 0.05$) in the gastrocnemius muscle of trained rats as compared with the sedentary animals (Fig. 1D).

Figures 2 and 3 show events occurring at the initial steps of the insulin signaling pathway response after stimulation with insulin in the intact rat. Sedentary and trained rats were injected with saline or insulin. Ninety seconds later, gastrocnemius muscle was extracted and homogenized as described in Methods. The extracts were immunoprecipitated with anti-IR (Fig. 2), anti-IRS-1 (Fig. 3A and C), and anti-IRS-2 antibodies (Fig. 3B and D), followed by SDS-PAGE under reducing conditions, and transferred onto

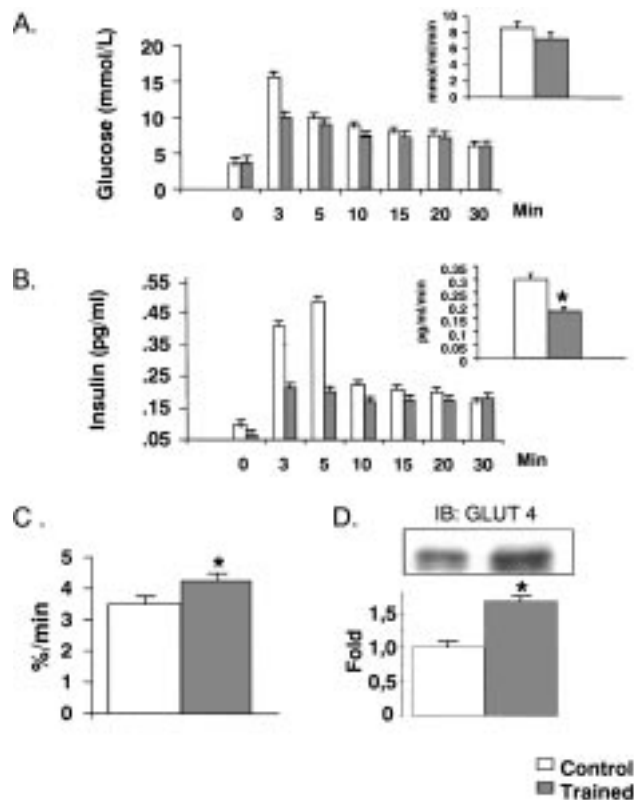


Figure 1 Effect of endurance training on glucose metabolism and GLUT-4 expression. (A) Glucose and (B) insulin concentrations during i.v. GTT. The respective areas under the curves for glucose (A) and insulin (B) are depicted in the insets. (C) Glucose disappearance rate in control and trained rats; the rate constant for plasma glucose disappearance (K_{ITT}) was calculated as described in Methods. (D) GLUT-4 protein content in muscle of sedentary (Control) and trained rats as detected by immunoblot (IB). Results are representative of the mean \pm s.d.; $n = 5$ for A, B and C, and $n = 6$ for D. * $P < 0.05$ compared with control.

nitrocellulose membranes. The transfers were then blotted using anti-phosphotyrosine (Figs 2, 3A and 3B), or anti-p85 PI3-kinase antibodies (Fig. 3C and D). There was no change in the insulin-induced tyrosine phosphorylation of the 95 kDa β -subunit of the IR of trained rats as compared with sedentary rats (Fig. 2). After endurance training, the insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2 increased by 1.8-fold ($P < 0.05$) and 1.5-fold ($P < 0.05$) respectively as compared with that in sedentary animals (Fig. 3A and B).

After insulin-induced phosphorylation, there is a high-affinity interaction between IRS-1 or -2 and the 85 kDa subunit of PI3-kinase, so that either protein can be co-precipitated by antibodies to the other protein. When blots obtained from immunoprecipitation experiments with anti-IRS-1 or anti-IRS-2 antibodies were re-blotted with antibodies against the 85 kDa subunit of PI3-kinase, a faint band was observed in the unchallenged state in both sedentary and trained

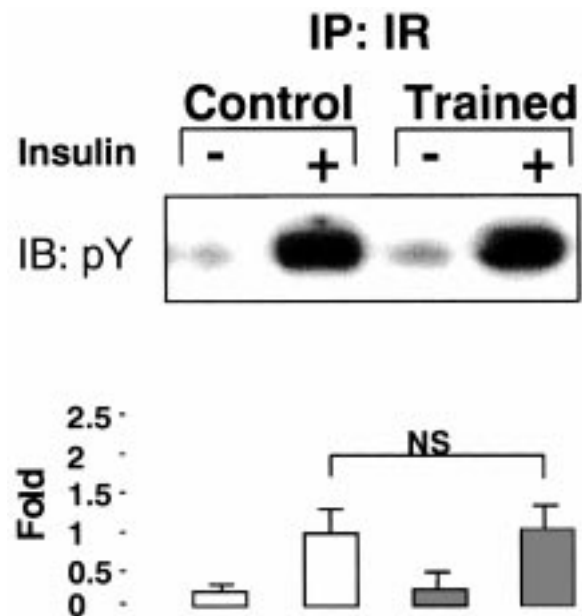


Figure 2 Insulin-stimulated tyrosine phosphorylation of IR in muscle of sedentary (Control) and trained rats. Immunoprecipitates (IP) collected with anti-insulin 95 kDa β -subunit antibodies were immunoblotted (IB) with anti-phosphotyrosine (pY) antibodies. Scanning densitometry was performed on autoradiographs from six experiments. Fold values are expressed as mean \pm s.d.

rats; after insulin stimulation the intensity of the bands increased in both groups. Comparison of the bands obtained from muscle extracts of rats stimulated by insulin revealed that the amount of PI3-kinase associated with IRS-1 increased by 2.3-fold ($P < 0.05$) and that associated with IRS-2 increased by 1.9-fold ($P < 0.05$) (Fig. 3C and D).

The effect of training upon PI3-kinase activity in muscle was determined by two different approaches: the measurement of IRS-1- or -2-associated-PI3-kinase capacity to incorporate phosphorus at the 3' position of inositol, and the assessment of insulin-induced Akt-1/PKB serine phosphorylation (as Akt-1/PKB is a downstream effector of PI3-kinase activity). IRS-1-associated PI3-kinase activity increased by 1.8-fold ($P < 0.05$) and IRS-2-associated PI3-kinase activity increased by 1.7-fold ($P < 0.05$) (Fig. 4). A low basal concentration of Akt-1/PKB, serine phosphorylation was present in anti-Ser 473 Akt-1 blots from sedentary and trained rats. After stimulation with insulin, there was a significant increase in Akt-1/PKB serine phosphorylation in trained rats (1.7-fold increase compared with sedentary rats, $P < 0.05$; Fig. 5).

The total amounts of IR, IRS-1, IRS-2 and Akt-1/PKB in gastrocnemius muscle were not affected by endurance training (Fig. 6), thus the observed changes in protein phosphorylation or association were an effect of training upon the activity of each of those elements

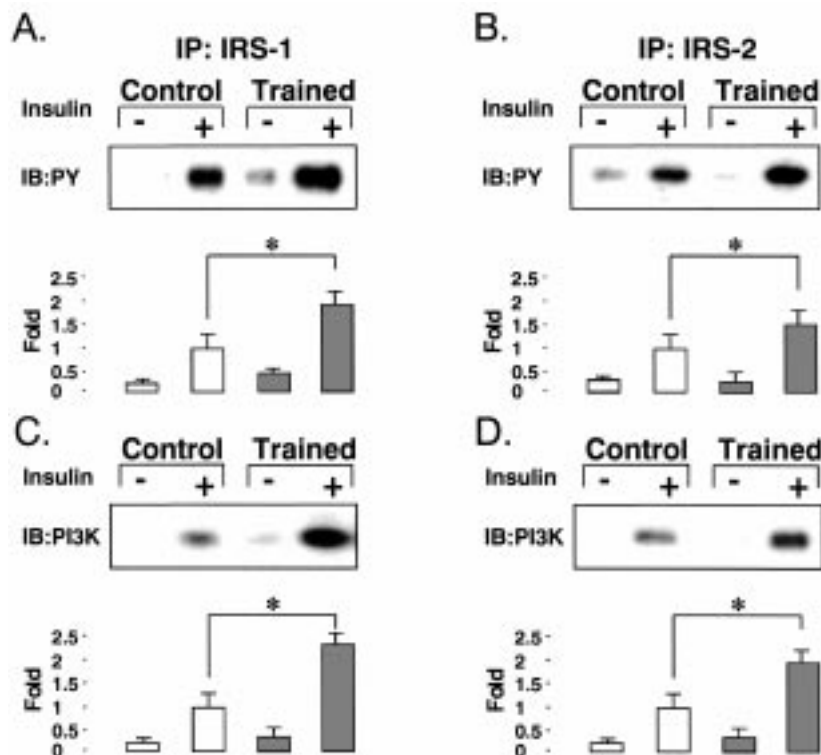


Figure 3 Insulin-stimulated tyrosine phosphorylation of IRS-1 or IRS-2, and IRS-1/ or IRS-2/PI3-kinase (PI3K) association in muscle of sedentary (Control) and trained rats. Immunoprecipitates (IP) collected with anti-IRS-1 (A and C), or anti-IRS-2 (B and D) antibodies were immunoblotted (IB) with anti-phosphotyrosine (PY) (A and B), or anti-p85 PI3-kinase (C and D) antibodies. Scanning densitometry was performed on autoradiographs from six experiments. Fold values are expressed as means \pm s.d. **P* < 0.05.

and their associated responses, and not a consequence of modulation of protein expression.

Discussion

Skeletal muscle responds to chronic exercise via a series of structural and functional adaptations. Muscle hypertrophy is a consequence of increased mitochondrial mass and an increased proportion of slow-oxidative myofibers (24). Activation of the mitogen-activated protein kinase pathway in exercise-stimulated muscle

has been demonstrated recently, and probably links mechanical activity to nuclear signaling and cellular growth (2). Exercise performed regularly leads to improved glucose homeostasis as a result of enhanced glucose tolerance and reduced insulin secretion. Apparently, increased muscle blood flow and increased insulin binding to its receptor are important mechanisms involved in the greater insulin sensitivity seen in trained humans and animals (3, 10). The final mediator of increased glucose clearance in trained individuals is enhanced GLUT-4 activity, as a result of increased GLUT-4 mRNA expression and protein

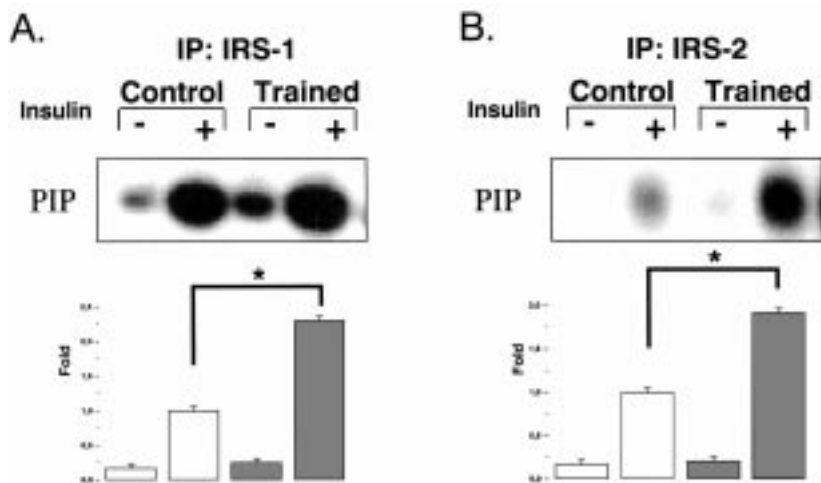


Figure 4 Insulin-stimulated IRS-1 or IRS-2 associated PI3-kinase activity. Immunoprecipitates (IP) collected with anti-IRS-1 or anti-IRS-2 antibodies were assayed for the associated PI3-kinase activity as described in Methods. Scanning densitometry of migrating phosphatidylinositol phosphate (PIP) was performed on autoradiographs from three sets of experiments. Fold values are expressed as means \pm s.d. **P* < 0.05.

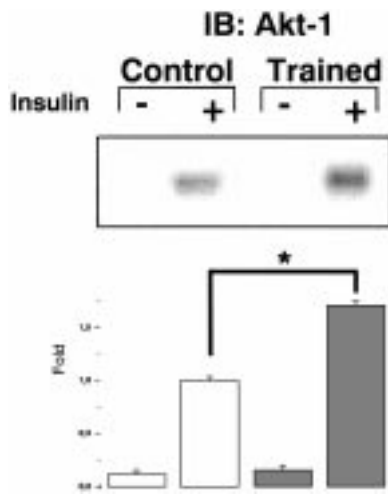


Figure 5 Insulin-stimulated serine phosphorylation of Akt-1/PKB. Muscle homogenates obtained from sedentary (Control) or trained rats were separated by SDS-PAGE. Immunoblotting (IB) was performed with anti-phosphoserine (Ser 473) Akt-1 antibodies. Scanning densitometry was performed on autoradiographs from five experiments. Fold values are expressed as means \pm S.D. * $P < 0.05$.

synthesis, and increased transporter translocation to the cell membrane, all of which lead to a greater velocity of glucose transport (3, 7, 10, 25, 26). However, the molecular mechanisms that link the extracellular phenomena of increased muscle blood flow and increased insulin binding to its receptor with those of increased glucose transporter expression and activity are unknown.

In the present work, we analyzed the early steps of insulin signaling in skeletal muscle of sedentary and trained rats. The endurance training model used matched previous descriptions: the rats had greater liver and muscle glycogen contents and responded to the tests performed (e.g. i.v. GTT and 15-min ITT) with an increased responsiveness to insulin. In order to avoid the acute effect of the last training bout, which is known to influence insulin action (27), all experiments were performed 48 h after the last training session. Therefore, the results obtained reflect the chronic adaptations induced by endurance training.

In the present model, endurance training did not affect the amounts of IR, IRS-1, IRS-2 and Akt-1/PKB in muscle. It did, however, promote a significant increase in GLUT-4 expression. Other authors have reported differences in specific protein contents in human athletes as compared with sedentary individuals (28). It is possible that species-specific factors may influence the rate of synthesis or degradation of proteins participating in insulin signaling.

Several reports have investigated the role of blood flow, insulin binding, IR protein and mRNA concentrations and IR turnover in the muscle of trained rats. Muscle blood flow is increased in endurance-trained humans and animals (3, 7, 10, 25, 26), as is insulin

binding to target tissues. Enhanced insulin binding is not a result of increased IR content, as shown in the present work and elsewhere (5), but probably reflects increased IR turnover. This conclusion is supported by the increased IR mRNA content seen in the muscle of trained rats (29). The amount of IRS-1 was investigated in short-term training and found to be reduced, whereas IRS-2 suffered no change (16). In long-term training, different results have been found. Nagasaki and co-workers (30) reported that endurance training was sufficient to decrease the rate of aging-dependent decrease in the muscle protein content of IRS-1 and PI3-kinase in rats. However, in another study the levels of IRS-1 mRNA were shown to increase in long-term trained rats (29). As there was no difference in the IRS-1 protein content in the present experiments, we suggest that an increased turnover of IRS-1, which might be dependent on duration and intensity of training, occurs in a manner similar to that observed for IR.

The kinase activity of the isolated IR towards itself and an exogenous substrate has been reported elsewhere (10). In that study, no increase in IR kinase activity was observed, supporting the present findings of no insulin-induced increase in IR phosphorylation in endurance-trained rats. Thus it is likely that increased IR turnover might be one of the causes of the subsequent intracellular events. Other possibilities are training-induced reduction in the activity of tyrosine phosphatases or other protein responsible for blunting the initial steps of insulin signaling.

The next step in insulin signaling involves the tyrosine phosphorylation of IRS-1 and IRS-2. As shown above, the amounts of IRS-1 and IRS-2 were unchanged in the muscle of trained rats. In contrast, the phosphorylation of IRS-1 and IRS-2 after stimulation with insulin increased significantly in those rats, compared with that in sedentary animals. As IRS-1 and IRS-2 are the main molecules linking insulin signaling to PI3-kinase activity and glucose transport, we examined the insulin-induced association of IRS-1 and -2 with the p85 subunit of PI3-kinase and found it to be increased in the muscle of trained rats. After IRS-1 or -2-PI3-kinase association, PI3-kinase is activated and can in turn activate Akt-1/PKB, a serine kinase with pleiotropic actions in several tissues (12). The activation of Akt-1/PKB is accompanied by an increase in its serine and threonine phosphorylation. The serine phosphorylation status of Akt-1/PKB after stimulation with insulin may reflect insulin-induced PI3-kinase activity. To prove this hypothesis, PI3-kinase activity assay was performed, and increases of 1.8-fold and 1.7 fold in IRS-1- and IRS-2-associated PI3-kinase respectively were detected. Moreover, an insulin-induced increase of 1.7-fold in Akt-1/PKB serine 473 phosphorylation was detected in muscle of endurance-trained rats as compared with sedentary controls. The mechanism by which Akt-1/PKB is activated by PI3-kinase is not fully understood. One

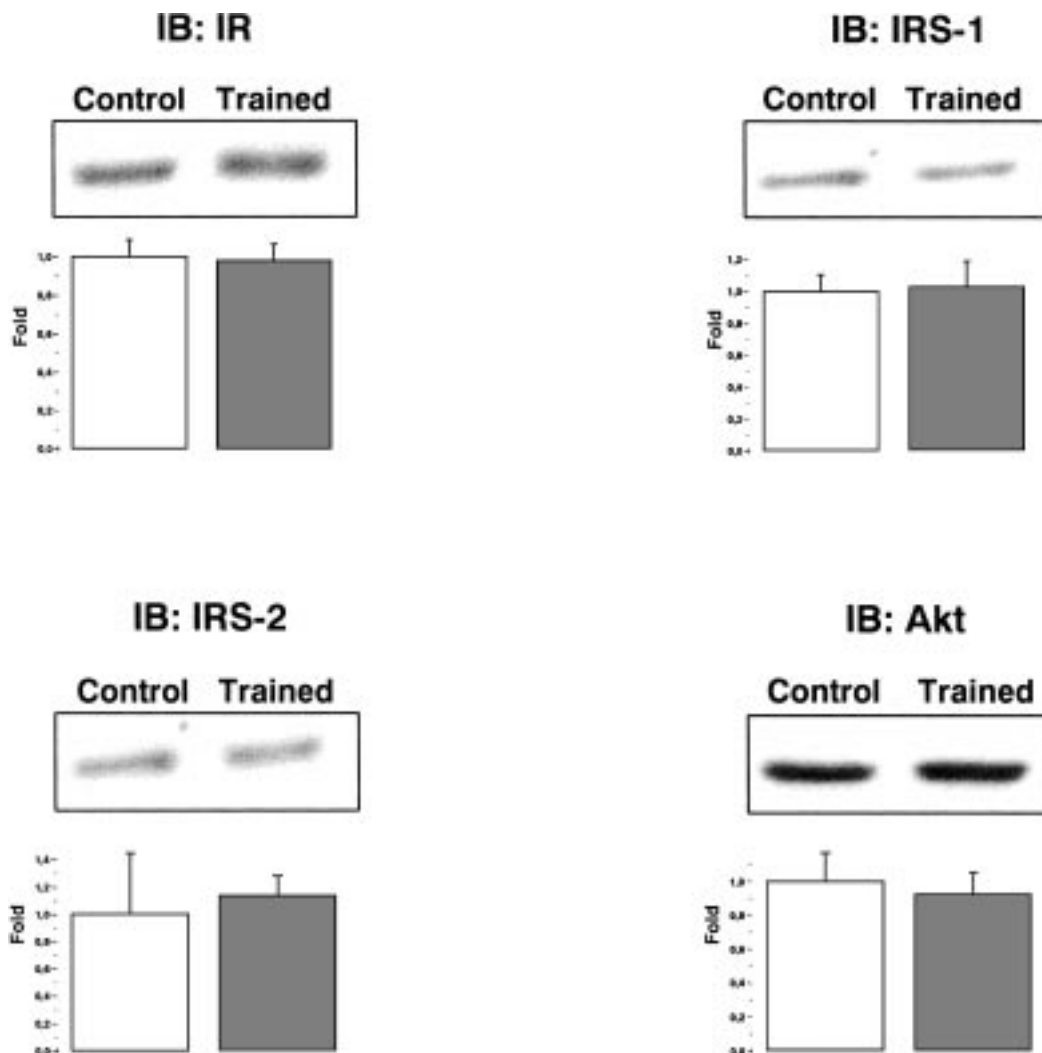


Figure 6 Effect of endurance training on IR, IRS-1, IRS-2 and Akt/PKB protein expression in muscle of rats. Total protein extracts from gastrocnemius muscle were prepared as described in Methods. Two hundred micrograms total protein from each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with specific anti-IR, -IRS-1, -IRS-2 and -Akt/PKB antibodies. Results are representative of the mean \pm s.d.; $n = 6$.

product of PI3-kinase action, PI(3,4)-biphosphate, stimulates Akt-1/PKB activity *in vitro* (12). It is supposed that, in resting cells, PI3-kinase and Akt-1/PKB are present in a cytoplasmic pool in an inactive form. After receptor tyrosine kinase activation, which leads to subsequent receptor autophosphorylation and recruitment of intracellular substrates, PI3-kinase is activated and recruited to the plasma membrane, where it will mediate the synthesis of PI(3,4,5)-triphosphate from PI(4,5)-biphosphate. The 3'-phosphorylated inositols act as targets for Akt-1/PKB, which is brought to the plasma membrane where it will be activated by the action of PI-dependent-kinases.

Thus the increase in the association between IRS-1 or -2 and PI3-kinase, and the increased PI3-kinase

activity after insulin infusion in regularly exercised rats may play a part in the increased responsiveness to insulin in these animals. Insulin increases glucose uptake into cells, partly through the translocation of GLUT-4 from intracellular compartments to the plasma membrane in muscle and adipose tissues (31, 32). Different experimental approaches have led to the conclusion that PI3-kinase is necessary for insulin-stimulated GLUT-4 translocation (32). Evidence from other sources has demonstrated a correlation between PI3-kinase activity and glycogen metabolism. Thus it is acceptable that the IRSs/PI3-kinase pathway may be linked to the activation of glucose transport and glycogen synthesis in muscle, and that an increase in this association in the muscle of trained rats may

have a role in the increased responsiveness to insulin observed in those animals.

In addition to the above-mentioned molecular phenomena, a morphofunctional adaptive change in fiber type may, also, be linked to the modulation of substrate responsiveness to insulin. The gastrocnemius muscle is composed of type I and type II (a and b) fibers. Endurance training is known to increase type I fiber mass (24). Thus the promotion of an increase in the relative proportions of slow twitch/fast twitch fibers may, at least partially, contribute to the modulation of the molecular events of insulin signaling demonstrated in the present study.

Compared with the recently studied effects of acute training upon insulin signaling (16, 17), the present data demonstrate that maintenance of training for longer periods stabilizes the amounts of proteins involved in insulin signaling and modulates the pattern of activation/function of those elements. Thus it seems that, under continuous and long-term training, the organism is adapted in such a way that the greatest response is achieved with no, or minimal, changes in effector or substrate concentrations. The differences between short- and long-term training may be a reflection of the adaptive changes occurring during the initial periods of training.

Conclusion

The enhanced glucose clearance achieved with endurance training involves modulation of the early steps of the insulin signaling pathway via a branch of the intracellular cascade directly linked to glucose transport.

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References

- American Diabetes Association Position Statement, Diabetes mellitus and exercise. *Diabetes Care* 1994 **17** 924–937.
- Aronson D, Violan MA, Dufresne SD, Zangen D, Fielding RA & Goodyear LJ. Exercise stimulates the mitogen-activated protein kinase pathway in human skeletal muscle. *Journal of Clinical Investigation* 1997 **99** 1251–1257.
- Hardin DS, Azzarelli B, Edwards J, Wigglesworth J, Maiano L, Brechtel G *et al.* Mechanisms of enhanced insulin sensitivity in endurance-trained athletes: effects on blood flow and differential expression of GLUT 4 in skeletal muscles. *Journal of Clinical Endocrinology and Metabolism* 1995 **80** 2437–2446.
- Hellenius M-LB, Brismar KE, Berglund BH & De Faire UH. Effects on glucose tolerance, insulin secretion, insulin-like growth factor 1 and its binding protein, IGFBP-1, in a randomized controlled diet and exercise study in healthy, middle-aged men. *Journal of Internal Medicine* 1995 **238** 121–130.
- Berger M, Hagg S & Ruderman NB. Glucose metabolism in perfused skeletal muscle. Interaction of insulin and exercise on glucose uptake. *Biochemical Journal* 1975 **146** 231–238.
- Ebeling P, Tuominen JA, Bourey R, Koranyi L & Koivisto VA. Athletes with IDDM exhibit impaired metabolic control and increased lipid utilization with no increase in insulin sensitivity. *Diabetes* 1995 **44** 471–477.
- Goodyear LJ, Hirshman MF & Horton ES. Exercise-induced translocation of skeletal muscle glucose transporters. *American Journal of Physiology* 1991 **261** E795–E799.
- Holloszy JO & Booth FW. Biochemical adaptations to endurance exercise in muscle. *Annual Reviews in Physiology* 1976 **38** 273–291.
- White MF & Kahn CR. The insulin signaling system. *Journal of Biological Chemistry* 1994 **269** 1–4.
- Dela F, Handberg A, Mikines KJ, Vinten J & Galbo H. GLUT 4 and insulin receptor binding and kinase activity in trained human muscle. *Journal of Physiology* 1993 **469** 615–624.
- Dela F, Larsen JJ, Mikines KJ, Ploug T, Petersen LN & Galbo H. Insulin-stimulated muscle glucose clearance in patients with NIDDM. *Diabetes* 1995 **44** 1010–1020.
- Franke TF, Kaplan DR, Cantley LC & Toker A. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 1997 **275** 665–667.
- Goodyear LJ, Giorgino F, Balon TW, Condorelli G & Smith RJ. Effects of contractile activity on tyrosine phosphoproteins and PI 3-kinase activity in rat skeletal muscle. *American Journal of Physiology* 1995 **268** E987–E995.
- Kitamura T, Ogawa W, Sakaue H, Hino Y, Kuroda S, Takata M *et al.* Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Molecular and Cellular Biology* 1998 **18** 3708–3717.
- Klippel A, Kavanaugh WM, Pot D & Williams LT. A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Molecular and Cellular Biology* 1997 **17** 338–344.
- Chibalin AV, Yu M, Ryder JW, Song XM, Galuska D, Krook A *et al.* Exercise-induced changes in expression and activity of proteins involved in insulin signal transduction in skeletal muscle: differential effects on insulin-receptor substrates 1 and 2. *Proceedings of the National Academy of Sciences USA* 2000 **97** 38–43.
- Wojtaszewski JE, Hansen BF, Gade J, Kiens B, Markuns JE, Goodyear LJ *et al.* Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 2000 **49** 325–331.
- Pimenta WP, Saad MJ, Paccola GM, Piccinato CE & Foss MC. Effect of oral glucose on peripheral muscle fuel metabolism in fasted men. *Brazilian Journal of Medical and Biological Research* 1989 **22** 465–476.
- Scott AM, Atwater I & Rojas E. A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. *Diabetologia* 1981 **21** 470–475.
- Bonora E, Zavaroni I, Alpi O, Pezzarossa A, Dall'Aglio E, Coscelli C *et al.* Influence of the menstrual cycle on glucose tolerance and insulin secretion. *American Journal of Obstetrics and Gynecology* 1987 **157** 140–141.
- Saad MJA, Carvalho CRO, Thirone ACP & Velloso LA. Insulin induces tyrosine phosphorylation of JAK2 in insulin-sensitive tissues of the intact rat. *Journal of Biological Chemistry* 1996 **271** 22100–22104.
- Carvalho CRO, Thirone ACP, Velloso LA & Saad MJA. Effect of captopril, losartan and bradykinin on early steps of insulin action. *Diabetes* 1997 **46** 1950–1957.
- Velloso LA, Folli F, Sun XJ, White MF, Saad MJA & Kahn CR. Cross-talk between the insulin and angiotensin signaling systems. *Proceedings of the National Academy of Sciences USA* 1996 **93** 12490–12495.
- Booth FW & Thomason RB. Molecular and cellular adaptation in response to exercise: perspectives of various models. *Physiology Reviews* 1991 **71** 541–585.

- 25 Ploug T, Stallknecht BM, Pedersen O, Kahn BB, Ohkuwa T, Vinten J *et al.* Effect of endurance training on glucose transport capacity and glucose transporter expression in rat skeletal muscle. *American Journal of Physiology* 1990 **259** E778–E786.
- 26 Ren JM, Samenkovich CF, Gulve EA, Gao J & Holloszy JO. Exercise induces rapid increases in GLUT 4 expression, glucose transport capacity, and insulin-stimulated glycosylation in muscle. *Journal of Biological Chemistry* 1994 **269** 14396–14401.
- 27 Terada S, Yokozeki T, Kawanaka K, Ogawa K, Higuchi M, Ezaki O *et al.* Effects of high-intensity swimming training on GLUT-4 and glucose transport activity in rat skeletal muscle. *Journal of Applied Physiology* 2001 **90** 2019–2024.
- 28 Yu M, Blomstrand E, Chibalin AV, Wallberg-Henriksson H, Zierath JR & Krook A. Exercise-associated differences in an array of proteins involved in signal transduction and glucose transport. *Journal of Applied Physiology* 2001 **90** 29–34.
- 29 Kim Y, Inoue T, Nakajima R, Nakae K, Tamura T, Tokuyama K *et al.* Effects of endurance training on gene expression of insulin signal transduction pathway. *Biochemical and Biophysical Research Communications* 1999 **254** 720–727.
- 30 Nagasaki M, Nakai N, Oshida Y, Li Z, Xu M, Obayashi M *et al.* Exercise training prevents maturation-induced decreases in insulin receptor substrate-1 and phosphatidylinositol 3 kinase in rat skeletal muscle. *Metabolism* 2000 **49** 954–959.
- 31 Richter EA, Garetto LP, Goodman MN & Ruderman NB. Muscle glucose metabolism in the rat: increased sensitivity to insulin. *Journal of Clinical Investigation* 1982 **69** 785–793.
- 32 Tanti JL, Grillo S, Gremeaux T, Coffey PJ, Van-Obberghen E & Le Marchand-Brustel Y. Potential role of protein kinase B in glucose transporter translocation in adipocytes. *Endocrinology* 1997 **138** 2005–2010.

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