Exercise does not alter subcellular localization, but increases phosphorylation of insulin-signaling proteins in human skeletal muscle

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Exercise does not alter subcellular localization, but increases phosphorylation of insulin-signaling proteins in human skeletal muscle. Am J Physiol Endocrinol Metab 290: E341–E346, 2006. First published September 27, 2005; doi:10.1152/ajpendo.00314.2005.—The subcellular localization of insulin signaling proteins is altered by various stimuli such as insulin, insulin-like growth factor I, and oxidative stress and is thought to be an important mechanism that can influence intracellular signal transduction and cellular function. This study examined the possibility that exercise may also alter the subcellular localization of insulin signaling proteins in human skeletal muscle. Nine untrained males performed 60 min of cycling exercise (~67% peak pulmonary O2 uptake). Muscle biopsies were sampled at rest, immediately after exercise, and 3 h postexercise. Muscle was fractionated by centrifugation into the following crude fractions: cytosolic, nuclear, and a high-speed pellet containing membrane and cytoskeletal components. Fractions were analyzed for protein content and subsequently influenced IRS-mediated signal transduction pathways (15). The majority of previous studies indicate that acute exercise or muscle contraction per se, does not influence the phosphorylation and activity of the insulin receptor, IRS-1, IRS-2, or other proximal proteins in the classical insulin signaling pathway in skeletal muscle (5, 6, 17, 23, 28). However, it should be noted that insulin-signaling proteins were measured only in skeletal muscle whole cell lysates. These previous studies do not rule out the possibility that, at the subcellular level, exercise may induce novel relocalization or spatial rearrangement of IRS proteins or other proteins in the insulin signaling pathway similar to insulin, (1–3, 7), IGF-I (18), and oxidative stress (19). It is possible that exercise-mediated changes in the subcellular localization of insulin signaling proteins may alter protein-protein interactions and subsequently influence IRS-mediated signal transduction pathways and cellular function in skeletal muscle (25). The aim of this study, therefore, was to determine whether an acute bout of exercise can result in changes in the subcellular localization of insulin signaling proteins in human skeletal muscle.

MATERIALS AND METHODS

Subjects. Nine healthy, active, but untrained males (21 ± 2 yr, 82.5 ± 11.5 kg, 181 ± 9 cm; means ± SE) volunteered to serve as subjects for the experiment. Subjects demonstrated no major contraindications to exercise, as determined by a detailed medical questionnaire. Before giving written consent to participate, subjects were asked to refrain from consuming alcohol, caffeine, or insulin-sensitizing drugs in the 24-h period before exercise. Subjects performed 60 min of cycling exercise (~67% peak pulmonary O2 uptake). Muscle biopsies were sampled at rest, immediately after exercise, and 3 h postexercise. Muscle was fractionated by centrifugation into the following crude fractions: cytosolic, nuclear, and a high-speed pellet containing membrane and cytoskeletal components. Fractions were analyzed for protein content and subsequently influenced IRS-mediated signal transduction pathways (15). The majority of previous studies indicate that acute exercise or muscle contraction per se, does not influence the phosphorylation and activity of the insulin receptor, IRS-1, IRS-2, or other proximal proteins in the classical insulin signaling pathway in skeletal muscle (5, 6, 17, 23, 28). However, it should be noted that insulin-signaling proteins were measured only in skeletal muscle whole cell lysates. These previous studies do not rule out the possibility that, at the subcellular level, exercise may induce novel relocalization or spatial rearrangement of IRS proteins or other proteins in the insulin signaling pathway similar to insulin, (1–3, 7), IGF-I (18), and oxidative stress (19). It is possible that exercise-mediated changes in the subcellular localization of insulin signaling proteins may alter protein-protein interactions and subsequently influence IRS-mediated signal transduction pathways and cellular function in skeletal muscle (25). The aim of this study, therefore, was to determine whether an acute bout of exercise can result in changes in the subcellular localization of insulin signaling proteins in human skeletal muscle.

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provided with both written and verbal information regarding the purpose, nature, and potential risks associated with the research study. The study protocol was approved by the Deakin University Human Research Ethics Committee.

**Pre-experimental protocol.** All subjects undertook an incremental exercise test to volitional fatigue on an electromagnetically braked cycle ergometer (Lode, Groningen, The Netherlands) to determine their peak pulmonary oxygen uptake (\(V\dot{O}_2\) peak). \(V\dot{O}_2\) peak was determined as the average oxygen uptake rate over the last minute of exercise before exhaustion (\(V\dot{O}_2\) peak = 47.1 ± 5.3 ml·kg\(^{-1}\)·min\(^{-1}\)). Subjects were instructed to refrain from physical activity, apart from that required for daily living, for 3 days before the trial. A standard diet (~14 MJ, 80% of total energy as carbohydrate) was provided for the subjects to consume the day before the exercise trial, and subjects also abstained from alcohol, tobacco, and caffeine. Subjects presented to the laboratory on the morning of the trial, having fasted for 10–12 h overnight.

**Experimental protocol.** All subjects undertook a single bout of cycling exercise for 60 min at 87 ± 5% \(V\dot{O}_2\) peak. After the exercise bout, subjects remained in the laboratory for 3 h and rested on a bed. Before the commencement of exercise, a catheter was inserted in a forearm vein for blood sampling. Blood was sampled at rest; at 10, 30, and 60 min of exercise; and then every 30 min during the 3-h recovery period. Muscle samples were obtained from the vastus lateralis using the percutaneous needle biopsy technique modified for suction (4) at rest (0 min), immediately after exercise (60 min), and after 3 h of recovery (240 min). Muscle samples were removed and immediately frozen in liquid nitrogen.

**Blood analysis.** Blood samples were transferred to lithium heparin tubes to prevent clotting and were then spun. The supernatant was removed and stored at −20°C for later analysis. Plasma samples were analyzed for glucose and lactate using an automated method (EML 105; Radiometer, Copenhagen, Denmark). Plasma insulin concentrations were measured by RIA (Linco Research, St. Charles, MO).

**Subcellular fractionation protocol.** Muscle samples were homogenized in a 1:4 w/vol ice-cold homogenization buffer (50 mM Tris, pH 7.8, 10 mM EDTA, pH 8.0, 100 mM NaF, 2 mM NaN\(_3\), 1 mM sodium pyrophosphate, 250 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) with a hand-held homogenizer (Polytron-Aggregate; Kinematica, Switzerland). Subcellular fractions were isolated by a method of differential centrifugation as previously described by Clark et al. (3), with the exception that SDS was used as a detergent to liberate proteins in the HSP rather than Triton X. IRS proteins have generally been considered to localize within cytosol, membrane, and cytoskeleton fractions (21), but recently, IRS proteins were detected in the nucleus, where they are thought to play an important role in the regulation of gene expression and cell growth and size (18, 26). Given that we have recently demonstrated in a separate study that exercise can induce translocation of proteins, in particular AMP-activated protein kinase, to a nuclear fraction in human skeletal muscle (11), a nuclear fraction was also isolated in the current study using the method previously described by McGee et al. (11). In summary, whole cell homogenates were initially spun for 10 min at 1,500 g (Beckman Ultracentrifuge). The resulting pellet was resuspended in 350 μl of homogenization buffer supplemented with detergents (10% glycerol, 1% Triton X-100, 50 mM-MgCl\(_2\)) to liberate nuclear components from contractile and fibrous tissue and kept on ice for 30 min before being spun for 5 min at 3,500 g (Biofuge, Heraeus, Germany). The resulting supernatant was removed, snap-frozen in liquid nitrogen, and stored at −80°C as the nuclear fraction. Supernatant from the 1,500-g step was transferred to new tubes and spun for 60 min at 200,000 g (Beckman Ultracentrifuge) to obtain a HSP. Supernatant was removed, snap-frozen, and stored at −80°C as the cytosolic fraction. The resulting HSP, which contained both cytoskeletal and membrane components, was resuspended in homogenizing buffer supplemented with detergent (1% SDS) to liberate proteins from the cytoskeleton and kept on ice for 60 min before being snap-frozen and stored at −80°C.

**Immunoblotting.** Total protein concentration for each fraction was determined (BCA Assay Kit; Pierce, Rockford, IL) using BSA as the standard. Proteins were separated and identified using SDS-PAGE. Samples (50–100 μg) were loaded onto 8% acrylamide SDS-PAGE gels with a molecular-weight marker (Precision Plus Protein Standards; Bio-Rad), before undergoing electrophoresis for ~60 min at 180 volts. After electrophoresis, proteins were transferred to a nitrocellulose membrane by using a wet transfer protocol for 60–90 min at 100 volts. Membranes were blocked for 1–2 h [5% BSA, 1× Tris-buffered saline (TBS), 0.01% Tween, and 0.1% NaN\(_3\)] before being washed overnight at 4°C for the following primary antibodies (1:1,000) in buffer (5% BSA in TBS, 0.5% Tween, and 0.1% NaN\(_3\)): polyclonal anti-insulin receptor β-subunit, anti-IRS-1 and -2, -p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase), anti-Akt/protein kinase B (PKB) and anti-glycogen synthase kinase-3 (GSK-3; Upstate Biotechnology), polyclonal phosphospecific antibodies for anti-IRS-1 phospho-Tyr612 (Biosource), anti-Akt phospho-Ser473, and anti-GSK-3α/β phospho-Ser21/9 (Cell Signaling). After incubation with the primary antibodies, membranes were washed in 1× TBS-Tween (TBST) and exposed to an appropriate horseradish peroxidase-labeled secondary antibody (1:2,000 in skim milk powder and 1× TBST) for 60 min. Membranes were exposed to chemiluminescence substrates (Perkin-Elmer Life Sciences, Boston, MA) and exposed on a Kodak Image Station 440CF (NEI Life Science Products). Specific bands of interest were identified and quantified using Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

**Calculation and statistical analysis.** Standards were included in all immunoblotting, and interassay variation was accounted for by normalizing data to control samples. All data are expressed as means ± SE. Statistical analysis was undertaken using a one-way ANOVA. When ANOVA revealed significant differences, further analysis was performed using the Student-Newman-Keuls post hoc test. The level of significance was set at \(P < 0.05\).

**RESULTS**

**Exercise.** Subjects exercised at a mean power output of 162 ± 18 watts for 60 min, requiring an oxygen uptake of 31.5 ± 4.5 ml·kg\(^{-1}\)·min\(^{-1}\) or 67 ± 5% \(V\dot{O}_2\) peak. The respiratory exchange ratio and ventilation averaged 0.96 ± 0.02 and 64.1 ± 2.71/min, respectively, during exercise. Plasma glucose levels did not change significantly from resting levels (5.6 ± 0.7 mmol/l) for the duration of exercise and the 3-h recovery period. Plasma insulin levels significantly decreased from resting levels of 76.7 ± 11.9 to 42.8 ± 5.8 pmol/l after 60 min of exercise, but, within 30 min after completion of exercise, plasma levels had returned to resting levels. Plasma lactate significantly increased from rest (1.3 ± 0.3 mmol/l) to an average during exercise of 5.6 ± 0.7 mmol/l. After exercise, plasma lactate levels rapidly returned to resting levels within the first 30 min of the recovery period.

**Subcellular localization.** Exercise resulted in no significant changes in the protein content of the insulin signaling proteins (insulin receptor, IRS-1 and -2, p85 subunit of PI 3-kinase, Akt, and GSK-3) in any of the crude skeletal muscle fractions (cytosol, HSP, and nuclear) either immediately after exercise or 3 h postexercise compared with rest (Figs. 1–3). Insulin.
receptor could not be detected by immunoblotting in the nuclear fraction (Figs. 1 and 2).

To determine whether exercise may influence the activation of insulin signaling proteins in the different skeletal muscle fractions, commercially available phosphospecific antibodies were employed. Exercise resulted in a small but nonsignificant increase in the phosphorylation of IRS-1 Tyr612 in the cytosol fraction (0 min, 3.83 ± 0.87; 60 min, 4.50 ± 0.73; 240 min, 6.15 ± 1.69), whereas in the HSP fraction IRS-1 phospho-Tyr612 was unchanged (0 min, 12.96 ± 2.34; 60 min, 12.76 ± 1.88; 240 min, 14.20 ± 2.82). IRS-1 phospho-Tyr612 was not detected in the nuclear fraction (Fig. 1). Phosphorylation of Akt Ser473 was increased significantly in the cytosol fraction immediately after exercise, although by 3 h after exercise phospho-Akt Ser473 had decreased toward preexercise values (Figs. 1 and 4). Exercise had no significant effect on the phosphorylation of Akt Ser473 in the nuclear fraction (Fig. 1). Phosphorylation of Akt Ser473 was increased significantly in the cytosol fraction immediately after exercise, although by 3 h after exercise phospho-Akt Ser473 had decreased toward preexercise values (Figs. 1 and 4). Exercise had no significant effect on the phosphorylation of Akt Ser473 in the nuclear fraction (Fig. 1). Phosphorylation of Akt Ser473 could not be detected in the HSP fraction. In the cytosol fraction, phosphorylation of GSK-3 Ser9/21 was increased (P < 0.05) by exercise and remained elevated (P < 0.05) 3 h after exercise (Figs. 1 and 4). There was a tendency for phospho-GSK-3 Ser9/21 to be increased immediately and 3 h after exercise in both the HSP and nuclear fraction.

DISCUSSION

The localization of IRS proteins within a cell is thought to play an important role in determining the magnitude and specificity of insulin-mediated cellular processes (21). IRS proteins have been shown to undergo changes in cellular localization in response to insulin stimulation (1–3, 7, 26), IGF-I, and oxidative stress (18, 19). In light of these previous findings, and that exercise or muscle contraction can influence many cellular functions that are similar to those also regulated by these various stimuli, the present study examined the novel hypothesis that exercise may alter the subcellular localization of IRS proteins and/or other proteins in the classical insulin signaling pathway.

With a technique of differential centrifugation, human skeletal muscle sampled at rest, immediately after an acute bout of moderate to intense cycling exercise, and 3 h postexercise was separated into the crude fractions (cytosol, nuclear, and HSP containing membrane and cytoskeletal components). The results demonstrate that exercise does not alter the content of IRS-1 and -2 or other proteins in the insulin signaling pathway, including insulin receptor, p85 subunit of PI 3-kinase, Akt, and GSK-3 in skeletal muscle subcellular fractions, either immediately or 3 h after exercise. Thus a change in the subcellular localization of insulin signaling proteins is an unlikely mechanism to influence downstream signal transduction pathways and subsequent cellular function in skeletal muscle.

Although the content of insulin signaling proteins did not change in the skeletal muscle fractions after exercise, it is possible that protein activation (phosphorylation and protein-protein interactions) may have been differentially altered by exercise in the subcellular fractions. Previous studies have demonstrated that activation of insulin receptor, IRS proteins, and associated PI 3-kinase activity are not influenced by exercise or muscle contraction, per se (5, 6, 17, 23, 28). However, in these studies, measurements were made in whole cell lysates where localized changes in the activation of individual proteins may be diluted and too small to be detected. By use of the percutaneous needle biopsy technique in humans, the size of a skeletal muscle biopsy sample is limited, and as such in the present study it was not possible to fully assess total tyrosine and/or serine phosphorylation and the activity of IRS proteins in each of the skeletal muscle fractions. However, with
the use of an antibody raised against a specific IRS-1 phosphorylation motif (Tyr612), a residue that is known to interact with the p85 regulatory subunit of PI 3-kinase, a small but nonsignificant increase in the phosphorylation of IRS-1 Tyr612 in the cytosol fraction could be detected immediately after exercise and 3 h after exercise, although no change was detected in the HSP fraction. In support of previous research (5, 6, 17, 23, 28), exercise, per se, does not appear to affect the activation of IRS-1, but it should be noted that IRS-1 is known to contain a number of phosphorylation sites, in addition to

Fig. 2. Protein content of IR, IRS-1, and IRS-2 in the cytosol, HSP, and nuclear fractions of human skeletal muscle before (0 min), immediately after (60 min), and 3 h after (240 min) cycling exercise. IR receptor was not detected in the nuclear fraction. Results are not directly comparable between the different fractions. Values are means ± SE (n = 9). Black bar denotes exercise.

Fig. 3. Protein content of the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase; p85), Akt, and GSK-3 in the cytosol, HSP, and nuclear fractions of human skeletal muscle before (0 min), immediately after (60 min), and 3 h after (240 min) cycling exercise. Results are not directly comparable between the different fractions. Values are means ± SE (n = 9). Black bar denotes exercise.
significantly increased immediately and 3 h after exercise. The cytosol fraction only, with GSK-3 serine phosphorylation Tyr612, that can act as on and/or off switches to recruit various stream target proteins in both the nucleus and cytoplasm, membrane and subsequently phosphorylates numerous downstream signaling proteins (20). Akt, or protein kinase B, is a serine/threonine kinase that, in response to various stimuli, is activated rapidly via translocation to the plasma membrane where it is phosphorylated. After activation, Akt dissociates from the membrane and subsequently phosphorylates numerous downstream target proteins in both the nucleus and cytoplasm, including GSK-3 (10). In the present study, the protein content of Akt and GSK-3 was not altered by exercise in any of the skeletal muscle fractions. However, in the cytosol fraction only, exercise significantly increased the phosphorylation of Akt and GSK-3 in response to exercise in the cytosol fraction only is unknown, although it may represent a level of biological control required to ensure regulation of specific exercise-mediated cellular functions. In rat skeletal muscle, contraction increases Akt Ser473 phosphorylation via a PI 3-kinase dependent mechanism (16), but Akt can also be activated in some cell systems by mechanisms that are independent of PI3-kinase, such as an increase in intracellular calcium (27). Whether this regulatory mechanism could also occur in contracting skeletal muscle, and perhaps more specifically in a cytosolic fraction only, is an area for further research. Alternatively, it cannot be completely ruled out that, in the HSP and nuclear fractions, Akt and GSK-3 were activated by exercise more rapidly and transiently than in the cytosol fraction and that methods employed in this study were not sensitive enough to detect significant increases in phosphorylation.

In the present study, GSK-3 serine phosphorylation was increased immediately and 3 h after exercise in the cytosol fraction only. One of the primary functions of GSK-3 is thought to be the regulation of glycogen synthase. An increase in GSK-3 serine phosphorylation deactivates the GSK-3 protein, which subsequently acts to increase glycogen synthase activity. However, in response to exercise, it is not entirely clear as to whether glycogen synthase is a direct physiological substrate of GSK-3 in skeletal muscle (14, 16, 24). GSK-3 may play a role in other cellular functions, including fuel metabolism, gene transcription, cell division, and survival. Interestingly, a study in rat skeletal muscle examining the subcellular localization and translocation of glycogen synthase in response to either high or low glycogen levels (manipulated through diet and exercise) could not detect glycogen synthase protein and activity in the cytosol fraction (12). Cellular localization and activation of glycogen synthase was not measured in the present study, and direct comparison with the study by Nielsen et al. (12) is further complicated by differences in the muscle fractionation techniques and exercise intervention employed. However, the potential differences in cellular localization and activation between GSK-3 and glycogen synthase highlight an important area for further research examining protein-protein interactions and regulation at the subcellular level to explain how exercise may mediate specific cellular responses in skeletal muscle.

In conclusion, an acute bout of moderate to intense cycling exercise can increase phosphorylation of downstream signaling proteins (Akt and GSK-3), specifically in the cytosolic fraction, but does not result in changes in the subcellular localization of IRS proteins or other proteins in the classical insulin signaling pathway in human skeletal muscle. As such, a change in the subcellular localization of insulin signaling proteins is an unlikely mechanism to influence downstream signal transduction pathways and subsequent cellular function in skeletal muscle after exercise.

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