Exercise-associated differences in an array of proteins involved in signal transduction and glucose transport

MEI YU,1,2 EVA BLOMSTRAND,2,3 ALEXANDER V. CHIBALIN,1,2 HARRIET WALLBERG-HENRIKSSON,1,2 JULEEN R. ZIERATH,1,2 AND ANNA KROOK1,2

1Department of Clinical Physiology, Karolinska Hospital, SE-171 76 Stockholm; 2Department of Physiology and Pharmacology, Karolinska Institute, SE-171 77 Stockholm; and 3Department of Sport and Health Sciences, Stockholm University College of Physical Education and Sports, SE-114 86 Stockholm, Sweden

Received 31 May 2000; accepted in final form 2 August 2000

Yu, Mei, Eva Blomstrand, Alexander V. Chibalin, Harriet Wallberg-Henriksson, Juleen R. Zierath, and Anna Krook. Exercise-associated differences in an array of proteins involved in signal transduction and glucose transport. J Appl Physiol 90: 29–34, 2001.—Vastus lateralis muscle biopsies were obtained from endurance-trained (running ~50 km/wk) and untrained (no regular physical exercise) men, and the expression of an array of insulin-signaling intermediates was determined. Expression of insulin receptor and insulin receptor substrate-1 and -2 was increased 4.7-fold (P < 0.05), whereas p38 mitogen-activated protein kinase expression was 32% lower (P < 0.001), respectively, in trained vs. untrained muscle. The downstream signaling target, Akt kinase, was not altered in trained subjects. Components of the mitogenic signaling cascade were also assessed. Extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase expression was 190% greater (P < 0.05), whereas p38 mitogen-activated protein kinase expression was 32% lower (P < 0.05), in trained vs. untrained muscle. GLUT-4 protein expression was twofold higher (P < 0.05), and the GLUT-4 vesicle-associated protein, the insulin-regulated aminopeptidase, was increased 4.7-fold (P < 0.05) in trained muscle. In conclusion, the expression of proteins involved in signal transduction is altered in skeletal muscle from well-trained athletes. Downregulation of early components of the insulin-signaling cascade may occur in response to increased insulin sensitivity associated with endurance training.

insulin receptor; insulin receptor substrate; GLUT-4; mitogen-activated protein kinase; citrate synthase

ENDURANCE EXERCISE TRAINING is associated with enhanced glucose tolerance and insulin action in healthy (11, 37) and insulin-resistant (23, 35, 41) people. Although exercise training directly leads to increased glucose disposal, this cannot be fully attributed to the effect of the last bout of exercise (9, 35). The molecular mechanism for enhanced glucose uptake with exercise training may be partly related to increased expression and activity of an array of key proteins known to regulate glucose metabolism in skeletal muscle (19). Increased expression of the insulin-responsive glucose transporter (GLUT-4) has been observed in response to exercise training, and this has been correlated with improved insulin action in skeletal muscle (10, 19, 23, 33). However, it is not known whether exercise training-associated improvements in glucose uptake are limited to increased GLUT-4 expression.

The intracellular signaling pathway by which insulin mediates glucose transport has been studied intensively. After insulin binds to the extracellular portion of the insulin receptor (IR), the intracellular tyrosine kinase activity of the receptor is activated, and several downstream substrates are phosphorylated (reviewed in Ref. 43). Important downstream substrates of the IR include the IR itself, as well as the IR substrates (IRS). To date, four different IRS molecules with different tissue distributions have been cloned (reviewed in Ref. 44). Tyrosine-phosphorylated IRS proteins act as docking proteins for signaling molecules containing Src homology 2 domains, including the 85-kDa regulatory subunit of phosphatidylinositol (PI) 3-kinase (43). PI3-kinase is a key signaling transducer in mediating downstream biological responses, including insulin-mediated GLUT-4 translocation and glucose transport (43, 44). Improvements in insulin sensitivity after exercise training may be related to changes in expression and/or activity of proteins involved in insulin signal transduction in skeletal muscle.

In skeletal muscle, both insulin and muscle contraction lead to activation of the mitogen-activated protein (MAP) kinase cascade (2, 13, 45). Activation of MAP kinase signaling pathways has been implicated in control of gene expression and protein synthesis (5, 7, 17). Several parallel MAP kinase pathways have been identified. The classical extracellular signal-regulated kinase (ERK) 1/2 pathway is associated with mitogenic responses, whereas p38 MAP kinase and stress-activated protein kinase integrate signals from diverse extracellular stimuli and/or various forms of cellular stress (7, 8). Whether habitual exercise training leads
to alterations in expression of various MAP kinase proteins is not known.

In skeletal muscle, insulin increases glucose transport by translocation of GLUT-4, the major glucose transporter expressed in skeletal muscle (24), from an intracellular pool to the plasma membrane (18). Muscle contraction is also a potent stimulus of GLUT-4 translocation (12, 32). Activation of 5'-AMP-activated kinase (15, 42), as well as changes in the level of cytoplasmic calcium levels (46, 47), leads to an insulin-independent increase in glucose transport activity and may be involved in the contraction response. The intracellular vesicles in which GLUT-4 resides appear to form a highly specialized compartment. The nature of this compartment and its trafficking pathway to the plasma membrane is still unresolved. Several glycoproteins are known to colocalize with GLUT-4 and translocate to the plasma membrane in an insulin-dependent manner (39). The insulin-regulated membrane aminopeptidase (IRAP) is one of the major proteins that colocalize with GLUT-4 and undergoes an insulin-dependent translocation to the cell surface (36, 40). Denervation downregulates GLUT-4 in skeletal muscle without affecting the level of expression of other known components of the corresponding vesicles (48), suggesting that muscle activity elicits a differential response in the level of expression of these proteins. Whether exercise training alters IRAP protein expression in a manner analogous to that of GLUT-4 is unknown.

We hypothesized that changes in expression of key proteins in the insulin signal-transduction pathway to glucose transport occur in skeletal muscle from people engaged in regular exercise training. Skeletal muscle biopsies were obtained from a trained and an untrained group of subjects, and protein expression of an array of key components of the insulin signal-transduction pathway was determined.

**MATERIALS AND METHODS**

**Materials.** The IR monoclonal CT3 antibody was raised against the COOH-terminal of the IR β-subunit and was a gift from Dr. Ken Siddle (Cambridge University, Cambridge, UK). The IRAP polyclonal antibody was raised against the COOH-terminal 16 amino acids (26) and was a gift from Dr. Susanna Keller (Dartmouth Medical School, Hanover, NH). All other antibodies were purchased from commercial sources, and the specific details regarding the generation of these antibodies are available from the suppliers. IRS-1 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY), IRS-2 polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY), GLUT-4 polyclonal antibody was purchased from Biogenesis (Poole, UK), and Akt kinase, ERK 1/2 MAP kinase, and p38 MAP kinase antibodies were purchased from New England Biolabs (Beverly, MA). These reagents have been valuable tools to detect protein expression of signaling intermediates and downstream effectors in human skeletal muscle (14, 30, 31, 45). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G was from Bio-Rad Laboratories (Richmond, CA). Reagents for enhanced chemiluminescence were from Amersham (Arlington Heights, IL). All other reagents were analytical grade (Sigma Chemical, St. Louis, MO).

**Subjects.** Seventeen healthy young male volunteers participated in the study. The study groups consisted of 11 habitual runners (trained) and 6 sedentary (untrained) controls. Subject characteristics for the habitually trained and untrained subjects are summarized in Table 1. Body mass index was similar between the trained and untrained subjects. Mean age was 10 yr greater in trained vs. untrained subjects; however, this difference was not statistically significant (P = 0.06). Subjects refrained from exercise training for 48 h before the biopsy sample was taken. The amount of exercise training was assessed by means of a questionnaire. The trained subjects reported participation in endurance running exercise of 2–10 bouts per week (47 ± 5 total km/wk) for not less than 2 mo before the investigation. Trained subjects reported an average marathon time (in h:min) of 3:45 (range 2:56–4:33), classifying these individuals among “well-trained amateur” runners, rather than “elite athletes.” The untrained subjects did not partake in regular sporting activities. After local anesthesia (mepivacaine chloride 5 mg/ml), an incision (5 mm long, 10 mm deep) was made in the skin and muscle fascia, and two muscle biopsies (20–100 mg) were obtained from the vastus lateralis portion of the quadriceps femoris by means of a Weil-Blakesley conchotome. Muscle tissue was immediately frozen and stored in liquid nitrogen until further analysis. One muscle biopsy was used for measurement of maximal activity of citrate synthase, and the other biopsy was used for protein expression studies. Informed consent was obtained from each subject, and the Ethical Committee at Karolinska Institute approved the study protocol.

**Table 1. Subject characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Trained</th>
<th>Untrained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>39 ± 4</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.5 ± 0.5</td>
<td>23.2 ± 0.9</td>
</tr>
<tr>
<td>Distance run per week, km</td>
<td>46 ± 5</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>Maximal citrate synthase activity, μmol·min⁻¹·g⁻¹ at 25°C</td>
<td>17.9 ± 1.2*</td>
<td>12.3 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.01 vs. untrained subjects.

**Determination of citrate synthase activity.** Citrate synthase activity was analyzed in muscle homogenates as described by Alp and co-workers (1). The muscles were weighed and homogenized in 10 vol of ice-cooled extraction buffer (50 mmol/l Tris, 5 mmol/l MgCl₂, 1 mmol/l EDTA, pH 8.2) using a ground-glass homogenizer. Citrate synthase activity was assessed spectrophotometrically. Oxaioacetate (0.35 mmol/l final concentration) was used as substrate.

**Protein expression studies.** Skeletal muscle biopsies (60–70 mg) were homogenized in ice-cold buffer (50 mmol/l Tris·HCl, 0.1% Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA, 50 mmol/l NaF, 5 mmol/l Na₂PO₄, 10 mmol/l glycerophosphate, 1 mmol/l Na₃VO₄, 1 μmol/l microcystin, 0.1% β-mercaptoethanol). Homogenates were rotated for 60 min at 4°C and subjected to centrifugation (12,000 g for 10 min at 4°C). Protein concentration of the resulting supernatant was determined by using a commercial kit (Bio-Rad, Richmond, CA). Aliquots of lysates (20 μg) were mixed with Laemmli sample buffer, and proteins were separated by SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in Tris-buffered saline, Tween-20 (TBST) (10 mmol/l Tris, 100 mmol/l NaCl, 0.02% Tween 20) containing 7.5% nonfat milk for 2 h at room temperature, washed with TBST for 10 min, and incubated with appropri-
ate primary antibody overnight at 4°C. The next morning, membranes were washed several times with TBST and incubated with appropriate secondary antibody for 1 h at room temperature. Membranes were then washed with TBST again and then subjected to enhanced chemiluminescence. Results were quantified by densitometry.

Statistics. Results are presented as means ± SE. Differences between trained and untrained subjects were determined by using Student’s t-test. Significance was accepted at P < 0.05.

RESULTS

Citrate synthase activity. Endurance exercise training is associated with large increases in the activity of enzymes of the citric acid cycle (20, 38). Thus we measured citrate synthase activity in the muscle biopsies. As expected, trained subjects had higher citrate synthase activity than untrained subjects (1.5-fold, P < 0.02), suggesting increased capacity for oxidative metabolism in skeletal muscle.

Effects of habitual exercise training on protein expression of IR, IRS-1, IRS-2, and Akt. Improved insulin sensitivity after exercise training is a well-established phenomenon; however, the changes that occur at the molecular level to account for improved insulin action are undefined. We hypothesized that habitual exercise was associated with an altered expression of proteins involved in intracellular transduction of the insulin signal. Protein expression of IR, IRS-1, and IRS-2, three critical molecules in the insulin signal-transduction cascade, was assessed. Interestingly, we noted a profound decrease in the expression of these early components of the insulin-signaling cascade. IR, IRS-1, and IRS-2 protein expression was 44% (P < 0.05, Fig. 1A), 57% (P < 0.001 Fig. 1B), 70% (P < 0.001, Fig. 1C) lower, respectively, in skeletal muscle from trained vs. untrained subjects. However, expression of the downstream kinase Akt was similar between the two groups (Fig. 1D).

Effects of habitual exercise training on ERK 1/2 and p38 MAP kinase protein expression. Several MAP kinase cascades are activated acutely by exercise in skeletal muscle (2, 13, 45). Whether habitual exercise training also leads to changes in protein expression of components of the MAP kinase cascades is not known. Total ERK expression was 190% greater (P < 0.05, Fig. 2A) in skeletal muscle from trained subjects compared
GLUT-4 and insulin-regulated aminopeptidase (IRAP) protein expression. Muscle biopsies were obtained from trained and untrained subjects as described in Fig 1. Top: representative immunoblot of GLUT-4 (A) or IRAP (B) protein in skeletal muscle from trained and untrained subjects. Bottom: data from trained (n = 11) and untrained (n = 6) subjects for GLUT-4 (A) and IRAP (B). Values are means ± SE in relation to mean untrained values. *P < 0.05 for trained vs. untrained subjects.

with the untrained group. In contrast, protein expression p38 MAP kinase was 32% lower in trained compared with untrained subjects (P < 0.05, Fig. 2B). Thus members of the MAP kinase signaling cascade are likely to undergo a differential regulation in response to habitual exercise training.

Effects of habitual exercise on GLUT-4 and IRAP protein expression. Exercise training is associated with increased insulin sensitivity. Exercise training is also associated with increased expression of GLUT-4 in skeletal muscle (23). Here we confirm earlier studies that GLUT-4 protein expression was increased in exercise-trained individuals. GLUT-4 expression was twofold greater in skeletal muscle from exercise-trained subjects (P < 0.05, Fig. 3A). GLUT-4 content was positively correlated with citrate synthase activity (r = 0.58 for all subjects; P < 0.02). We next assessed whether the increase in GLUT-4 was accompanied by changes in protein expression of IRAP, a major constituent of the GLUT-4 vesicle. IRAP expression was 4.7-fold greater in skeletal muscle from trained subjects (P < 0.05, Fig. 3B). Thus habitual exercise is associated with a coordinated upregulation of both GLUT-4 and IRAP in skeletal muscle.

DISCUSSION

Exercise is an important regulator of protein synthesis and gene transcription in skeletal muscle (3, 4). Chronic exercise training leads to changes in skeletal muscle mitochondrial mass and muscle oxidative capacity (20, 21, 38) and enhances whole body insulin sensitivity (11, 19, 35, 37, 41). Here we provide molecular evidence that habitual exercise training is associated with a differential protein expression of several components of the signal-transduction pathway in human skeletal muscle. Importantly, these changes are noted in skeletal muscle from well-trained endurance athletes. Whether more profound changes in the expression of these proteins can be observed in elite athletes or athletes engaged in higher volume training is not known.

GLUT-4 protein expression is increased in human skeletal muscle after exercise training (10, 19, 23). We noted a weak but positive correlation between muscle citrate synthase activity and GLUT-4 protein expression (r = 0.58 for all subjects), providing evidence for an exercise “dose-response” effect. IRAP is a major component of the GLUT-4 vesicle (25, 26), and, like GLUT-4, IRAP translocates to the cell surface in response to insulin (25, 36, 40). Similar to GLUT-4 expression, IRAP protein expression also increased in skeletal muscle from exercise-trained subjects. The physiological function of IRAP and the role it plays in insulin action are presently unknown. IRAP cleaves several peptide hormones in vitro (16). In insulin-treated isolated fat cells, concomitant with IRAP’s appearance at the cell surface, aminopeptidase activity toward extracellular substrates increases (16, 36). The parallel increase in expression of both GLUT-4 and IRAP noted in this study indicates that a coordinated upregulation of the components of the insulin-sensitive intracellular vesicles may occur in response to exercise training. These changes in GLUT-4 and IRAP expression might have been even greater if the subjects in the present study were better matched for age.

Expression of the IR was decreased in skeletal muscle from the trained subjects. This was accompanied by a decrease in expression of IRS-1 and IRS-2, two key downstream targets of the IR in skeletal muscle. These results were unexpected given that acute and long-term exercise training are associated with enhanced whole body insulin sensitivity (11, 19, 23, 34, 37, 41). Furthermore, habitual and short-term (7-day) exercise training have both recently been reported to be associated with enhanced insulin-stimulated PI3-kinase activity (22, 29).

We have performed a cross-sectional study; thus our trained subjects may be genetically predisposed to reduced protein expression of the insulin-signaling machinery. Alternatively, because the trained subjects are approaching middle age and are 10 yr older than the untrained subjects (P = 0.06), the possibility remains that they are more insulin resistant compared with younger, similarly trained subjects. Thus the decrease in some of the insulin-signaling molecules studied may be explained by advanced age in the trained subjects. However, short-term exercise training in rats is also associated with decreased IRS-1 protein expression (6), consistent with our present findings in humans. Despite reduced IRS-1 expression, insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI3-kinase activity were increased in exercise-trained skeletal muscle (6). Interestingly, expression of Akt kinase, a more distal insulin-signaling intermediate, was similar between trained and untrained muscle (present study and Ref. 6). Thus in-
creased insulin action in skeletal muscle after exercise training is associated with enhanced insulin signal transduction, concomitant with decreased protein expression of early components of the insulin-signaling cascade.

Protein expression of early components of the insulin-signaling cascade were reduced in trained subjects. Thus repeated exercise may be associated with either increased degradation or decreased synthesis of these components of the insulin-signaling machinery. Gene expression of the insulin-signaling pathway intermediates (IRS-1, ERK1 MAP kinase, PI3-kinase, GLUT-4, p70 S6 kinase, and Ras mRNA) increased in rodent skeletal muscle after 9 wk of treadmill training (27, 28). Taken together with our data, these findings suggest that IRS-1 is likely to undergo protein degradation in response to exercise training, whereas ERK1 and GLUT-4 are likely to undergo increased protein synthesis. In cultured cells, hyperinsulinemia leads to IRS-1 degradation (34). However, hyperinsulinemia is unlikely to account for the training-associated reduction in IR and IRS-1 and IRS-2 protein content, because exercise is known to lower insulin levels (37). Rather, the reduced protein expression may occur as a negative-feedback mechanism in response to increased insulin signaling (22, 29), and this may be a means to prevent excessive glucose uptake into skeletal muscle.

The MAP kinase family forms a major and ubiquitous intracellular signaling system that regulates cell growth, differentiation, and cell survival (8). Expression of ERK 1/2 MAP kinase was profoundly increased in skeletal muscle from trained subjects. In contrast to our results for ERK 1/2, p38 MAP kinase expression was decreased in skeletal muscle from trained subjects. Muscle contraction through exercise directly leads to the activation of several MAP kinase cascades (2, 13, 45). In a previous study, our laboratory reported a greater increase in ERK 1/2 phosphorylation than in p38 MAP kinase phosphorylation, in response to 30 min of bicycle exercise (45). Activation of MAP kinase signaling is a candidate mechanism whereby muscle contraction may signal to alterations in gene expression (2, 13). Habitual exercise may provide a physiological stimulus to regulate expression of different MAP kinase family members.

In summary, we provide molecular evidence for differential effects of habitual exercise on the expression of an array of key signaling proteins in skeletal muscle. Regular exercise training is associated with a striking downregulation of early components of the insulin-signaling cascade, concomitant with a profound upregulation of GLUT-4 and IRAP in skeletal muscle. Furthermore, components of the mitogenic signaling cascades appear to play specialized roles in modulating exercise adaptations on gene expression, because ERK 1/2 MAP kinase expression was increased in trained muscle, whereas p38 MAP kinase expression was decreased. Downregulation of the early components of the insulin-signaling cascade may be a result of increased protein degradation in response to exercise. This may serve as a feedback mechanism to prevent excessive glucose entry into skeletal muscle and hyperglycemia.

We thank Drs. Bjorn Ekblom and Jan Henriksson for contributions to study, Dr. Susanna Keller for helpful comments on the manuscript, and the volunteers.

This study was supported by grants from the Swedish Medical Research Council (12699, 12679, 9517), Thurings Foundation, Wibergs Stiftelse, Magnus Bergwalls Stiftelse, Tore Nilsens Stiftelse, the Novo-Nordisk Foundation, Harald and Greta Jeanssons Stiftelse, the Swedish Diabetes Association, the Swedish National Centre for Research in Sports, and the Foundation for Scientific Studies of Diabetology.

REFERENCES

19. Hjeltnes N, Galuska D, Bjornholm M, Aksnes AK, Lannem