Changes in insulin-stimulated glucose transport and GLUT-4 protein in rat skeletal muscle after training

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Division of Health Promotion, National Institute of Health and Nutrition, Toyama 1-23-1, Shinjuku-City, Tokyo 162; and Institute of Health and Sport Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba-City, Ibaraki 305, Japan

Kawanaka, Kentaro, Izumi Tabata, Shigeru Katsuta, and Mitsuru Higuchi. Changes in insulin-stimulated glucose transport and GLUT-4 protein in rat skeletal muscle after training. J. Appl. Physiol. 83(6): 2043–2047, 1997.—After running training, which increased GLUT-4 protein content in rat skeletal muscle by <40% compared with control rats, the training effect on insulin-stimulated maximal glucose transport (insulin responsiveness) in skeletal muscle was short lived (24 h). A recent study reported that GLUT-4 protein content in rat epitrochlearis muscle increased dramatically (−2-fold) after swimming training (J. M. Ren, C. F. Semenovich, E. A. Gulve, J. Gao, and J. O. Holloszy. J. Biol. Chem. 269, 14396–14401, 1994). Because GLUT-4 protein content is known to be closely related to insulin-stimulated glucose transport in the presence of a maximally stimulating concentration of insulin (2 mU/ml) were examined by using incubated epitrochlearis muscle preparation. Swimming training increased GLUT-4 protein content and insulin responsiveness by 87 and 85%, respectively, relative to age-matched controls when examined 24 h after swimming training. Therefore, we examined this possibility. Male Sprague-Dawley rats swam 2 h/day for 5 days with a weight equal to 2% of body mass. Approximately 18, 42, and 90 h after cessation of training, GLUT-4 protein concentration and 2-[1,2-3H]deoxy-D-glucose transport in the presence of a relatively high level and still remains higher than control level for >24 h after swimming training. Therefore, we examined this possibility. Male Sprague-Dawley rats swam 2 h/day for 5 days with a weight equal to 2% of body mass. Approximately 18, 42, and 90 h after cessation of training, GLUT-4 protein concentration and insulin responsiveness were still higher by 52 and 51%, respectively, in muscle from trained rats compared with control. GLUT-4 protein concentration and insulin responsiveness in trained muscle returned to sedentary control level within 90 h after training. We conclude that 1) the change in insulin responsiveness during detraining is directly related to muscle GLUT-4 protein content, and 2) consequently, the greater the increase in GLUT-4 protein content that is induced by training, the longer an effect on insulin responsiveness persists after the training.

EXERCISE TRAINING IMPROVES whole body insulin action on glucose disposal (13, 16). Skeletal muscle is the major site for insulin-stimulated glucose disposal (3). Because the rate-limiting step in glucose utilization in skeletal muscle is glucose transport across the sarcolemma under most physiological conditions (18, 25), improvement in whole body glucose disposal by exercise training is considered to be due to the increased insulin-stimulated glucose transport in skeletal muscle (4, 17). Furthermore, increased GLUT-4 protein, the major glucose transporter isoform in skeletal muscle, is thought to be one of the major mechanisms through which exercise training improves insulin-stimulated glucose transport in skeletal muscle (20, 22).

Several previous studies reported that the training-induced improvement in insulin-stimulated maximal glucose transport (insulin responsiveness) or uptake in rat skeletal muscle was short lived (24 h) and disappeared within 48 h (4, 7, 10, 11). In previous studies, the rats were trained by means of treadmill running (velocity, ~30 m/min; duration, 2 h/day; frequency, 5 or 6 days/wk; training period, 10–16 wk). This training protocol induced a relatively small (<40%) increase in GLUT-4 protein content in hindlimb muscles 24 h after training (4). Recently, Ren et al. (21) have reported a more dramatic increase in GLUT-4 protein content in rat epitrochlearis muscle (by ~2-fold) 24 h after prolonged short-term swimming training. Because GLUT-4 protein content is considered to be closely related to insulin responsiveness in skeletal muscle (8, 9), we thought it possible that the training effect on insulin responsiveness may remain for >24 h after swimming training if GLUT-4 protein content decreases gradually from a relatively high level and still remains higher than control level for >24 h after swimming training. Therefore, we swam the rats for 2 h/day with a weight equal to 2% of body mass for 5 days. Consequently, we found that insulin responsiveness and GLUT-4 protein content in rat epitrochlearis muscle increased by ~85% at 18 h after training and still remained ~50% higher than that observed in control at 42 h after training.

METHODS

Animal care and exercise program. Four-week-old male Sprague-Dawley rats (Crea Japan, Tokyo, Japan) with initial body weights of 80–90 g were used for this study. All animals were housed in rooms lighted from 7 AM to 7 PM and were maintained with ad libitum feeding on standard chow and water. Room temperature was maintained at 20–22°C.

One hundred eighty rats were randomly assigned to either a sedentary control or 5-day swimming-training group. Training-group rats swam 2 h/day in four 30-min bouts separated by 5 min of rest. After the first 30-min bout, a weight equal to 2% of body weight was tied to the body of the rat. The rats swam with the weight attached for the remaining three exercise bouts. All rats swam in a barrel filled to a depth of 50 cm and an average surface area of 190 cm²/rat. The rats performed the above swimming protocol once a day for 5 days. Training was started at 3:30 PM and ended at 6:00 PM every day. Approximately 16–20 (18 h), 40–44 (42 h), and 88–92 h (90 h) after the last bout of exercise, groups of trained
and control rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Food intake for all rats was restricted to 8 g from 7:00 PM on the last day before the experiment. All rats ate all 8 g of food. After that, epitrochlearis muscles were dissected out to measure glucose transport activity in vitro and GLUT-4 protein content. Muscle glucose transport activity and GLUT-4 protein content were determined on different rats, which were trained together, and sampled at the same time. At each time point during detraining, each group of swimming rats was compared with age-matched control rats. Sixteen to 20 h are long enough to permit the acute effect of exercise on insulin responsiveness to wear off (2).

Muscle preparation. Because epitrochlearis muscle is a thin muscle (<2 mm), it can be incubated intact and used to measure glucose transport in vitro (24). Additional epitrochlearis muscles were trimmed free of connective tissue and frozen for subsequent measurements of GLUT-4 protein concentration (6) and citrate synthase activities (23).

Muscle incubations. After dissection, epitrochlearis muscles were allowed to recover for 30 min in 3 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHB) containing 8 mM glucose and 32 mM mannitol. Epitrochlearis muscles to be used for measurement of insulin-stimulated glucose transport activity were placed in 3 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHB) containing 8 mM glucose, 32 mM mannitol, 0.1% radioimmunoassay-grade bovine serum albumin, and 2 mM/ml insulin (this concentration of insulin stimulates glucose transport to maximally) and were incubated with shaking at 35°C for 30 min. Control muscles were incubated under the same conditions. The gas phase in the flasks was 95% O2-5% CO2.

Measurement of glucose transport activity. Glucose transport activity was measured by using the glucose analog 2-deoxy-o-glucose (2-DG) and the procedure of Young et al. (24). After the initial incubation periods, the muscles were transferred to flasks containing 3 ml of KHB with 40 mM mannitol and insulin, if present during the previous incubation period, and were incubated with shaking for 10 min at 29°C to remove glucose. The muscles were then incubated for 20 min at 29°C in 2 ml of KHB containing 1 mM 2-[1-14C]-deoxy-o-glucose (1.5 mCi/mmol) and 39 mM o-[1,13C]-mannitol (8 µM/mmol) (Moravek Biochemicals), in the absence or presence of insulin. The gas phase in the flasks during both the rinse and incubation periods was 95% O2-5% CO2. After incubation, the muscles were blotted briefly on filter paper dampened with incubation medium, trimmed, and frozen in liquid N2. The extracellular space and intracellular 2-DG concentrations were determined as described previously (24). The samples were weighed and homogenized in 0.3 M perchloric acid. The remainder of the homogenate was centrifuged at 1,000 g. Aliquots of the muscle extracts and of the incubation media were placed in scintillation vials containing 2 ml of Aquasol-2 (NEN) and were counted in a liquid scintillation counter with channels preset for simultaneous 14C and 3H counting. The amount of each isotope present in the samples was determined, and this information was used to calculate the extracellular space and the intracellular concentration of 2-DG. The intracellular water content of the muscles was calculated by subtracting the measured extracellular-space water from total muscle water. Glucose transport activity is expressed as micromoles of 2-DG per milliliter intracellular water per 20 min.

Measurement of immunoreactive GLUT-4 protein. Epitrochlearis muscles were homogenized with a glass homogenizer (Kontes, Vineland, NJ) in 9 ml of buffer containing 0.25 M sucrose, 1 mM EDTA, and 10 mM tris(hydroxymethyl)amino-methane (Tris)-HCl, pH 7.5, at 4°C. The homogenate thus obtained was centrifuged at 175,000 g for 60 min, at 4°C. This pellet (the membrane fraction) was suspended in 490 µl of buffer containing 1 mM EDTA and 10 mM Tris-HCl, pH 7.5, at 4°C, and blended vigorously in a Vortex mixer until the visible pellets were completely dispersed. Then, this solution was solubilized by adding 10 µl of 0.35 M (10% wt/vol) sodium dodecyl sulfate (SDS), mixed well in a Vortex mixer, and kept for 10 min at room temperature. This solution was transferred to a microcentrifuge tube and centrifuged at 10,000 g for 15 min to remove unsolubilized materials. These solubi-

lized membranes were used for the assay of protein and GLUT-4 protein concentration. In a previous study (6), it was shown that almost all GLUT-4 protein was recovered in this procedure.

GLUT-4 protein was assayed as described by Ezaki et al. (6). The solubilized membranes were incubated for 10 min at 37°C in a solution containing 2.5% SDS, 75 mM dithiothreitol, 1.7 M (12.5% vol/vol) glycerol, 361 mM (0.025% wt/vol) bromophenol blue, and 12.5 mM Tris-HCl, pH 7.0. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (14) with a 0.56 M (4% wt/vol) stacking gel and a 1.4 M (10% wt/vol) resolving gel. Immunoblotting of electrophoresis gels was performed as described previously (5). Proteins in the gels were electrophoretically transferred to polyvinylidene difluoride sheets (immobilon, Millipore, Bedford, MA) in a transfer buffer. The sheets were incubated successively with antibodies to glucose transporters for 24 h and [125I]-labeled protein A for 24 h at 4°C. Autoradiography was performed with Kodak XAR film (Rochester, NY) at −70°C for 4–12 h. To quantify the glucose transporters, we cut out pieces of sheet containing the GLUT-4 protein and counted radioactivity in a gamma counter. The background was estimated by counting a region with no labeled band and then subtracting. To compare the amount of transporters in different sheets, we applied some samples, which were from age-matched control muscles excised the day after 5 days of training, to each gel as pooled control, and radioactivity of the corresponding bands was normalized.

Statistics. All values are expressed as means ± SE. Statistical comparisons of the groups were made by two-way analysis of variance, and individual groups were compared with means comparisons test (Super ANOVA, Abacus Concepts, Berkeley, CA). The correlation analysis was done with Spearman’s test. Statistical significance was defined as P < 0.05.

RESULTS

Glucose transport activity and GLUT-4 protein content. Eighteen hours and 42 h after 5 days of training, maximally insulin-stimulated 2-DG transport (insulin responsiveness) was 85 and 51% higher in muscles from trained rats compared with age-matched controls, respectively (P < 0.01; Fig. 1). The increases in GLUT-4 protein relative to age-matched controls at 18 and 42 h after 5 days of training (87 and 52%; P < 0.01, respectively) coincided with the increments in insulin responsiveness. Ninety hours after 5 days of training, there were no significant differences in insulin responsiveness and GLUT-4 protein concentration in muscles from trained rats and age-matched control (Figs. 1 and 2). When 2-DG transport was expressed relative to muscle wet weight, the results were similar to that described above (data not shown).
A significant positive correlation ($r = 0.98$, $P < 0.05$) was found for the insulin responsiveness and GLUT-4 protein content in muscle from trained and control rats (Fig. 3). When 2-DG transport was expressed relative to muscle wet weight, the similar relationship was observed (data not shown).

**Enzyme activity.** At 18 h after 5 days of training, citrate synthase activity was 48% increased compared with the age-matched control ($P < 0.01$; Table 1). This increase of citrate synthase activity was still present 90 h after the exercise was stopped (Table 1).

**DISCUSSION**

Swimming training increased insulin responsiveness and GLUT-4 protein content in rat epitrochlearis muscle by −85% relative to age-matched controls when examined 18 h after the rats stopped training (Figs. 1 and 2). Forty-two hours after the training, insulin responsiveness and GLUT-4 protein content were still higher by ∼50% in the muscle from trained rats (Figs. 1 and 2). This result shows that the training effects on insulin responsiveness and GLUT-4 protein content persists for up to 42 h after training was stopped in our study.

Previous studies showed that the effect of treadmill running training on insulin responsiveness in rat hind-limb muscle disappeared within 48 h after training (4, 7, 10, 11). These results differ somewhat from our results. In previous studies, the rats were trained by means of treadmill running (velocity, ∼30 m/min; duration, 2 h/day; frequency, 5 or 6 days/wk; training period, 10–16 wk). This training protocol increased muscle GLUT-4 protein content by only ∼37% in both red gastrocnemius and plantaris or by ∼26% in white gastrocnemius compared with sedentary level when examined 48 h after the training. On the other hand, in our study, the rats were trained by means of swimming
for 2 h/day with a weight equal to 2% of body mass. This
training protocol increased GLUT-4 protein content in
rat epitrochlearis muscle by 52% at 42 h after the
training (Fig. 2). As previously reported, total GLUT-4
protein content has been considered to be one of the
determining factors of insulin responsiveness in skeletal
muscle (8, 9). Therefore, we consider that the
difference between previous studies and our study can
be explained by the difference in the magnitude of the
increase in GLUT-4 protein content at ~48 h after training.
We believe it might be possible that the difference in insulin responsiveness between the trained and control groups 48 h after the training did not attain statistical significance because of the relatively small difference in GLUT-4 protein content between muscles from trained and control animals in previous studies.

We showed that the change in insulin responsiveness after training was accompanied by a parallel change in
GLUT-4 protein content (Fig. 3). Therefore, these results also suggest that the change in insulin responsiveness in rat epitrochlearis muscle with detraining is due to the change in total GLUT-4 protein content. However, it was reported in a previous study by Etgen et al. (4) that the magnitude of the increase in insulin responsiveness 24 h after training was greater than could be explained solely by the increase in GLUT-4 protein content. In their study, rats were fasted after the last exercise bout until being perfused on the next day, whereas, in the present investigation, rats were provided 8 g of chow after the last exercise session until measurement on the next day. Because restriction of carbohydrate after acute exercise amplifies insulin responsiveness (2), the difference in magnitude of the increase in insulin responsiveness on the next day after training between the study of Etgen et al. (4) and our study might be explained by the difference in the feeding schedule before glucose transport evaluation after the last bout of exercise. In fact, Etgen et al. actually showed that when rats were fed after the last bout of exercise training, insulin responsiveness in red gastrocnemius muscle was lower in fed trained rats than in fasted trained rats 24 h after training. Therefore, fasting after the last exercise bout may partially explain the greater increase in insulin responsiveness for a given increase in GLUT-4 protein content within 24 h after training.

In addition to the difference in protocols discussed above, several differences in the method for measuring glucose transport between our study and that of Etgen et al. (4) may explain the higher glucose transport for a given increase in GLUT-4 protein content in their study 24 h after training. First, in the present study, we measured glucose transport by using an in vitro incubated muscle preparation, whereas the hindlimb perfusion technique was used in the study by Etgen et al. Kern et al. (12) reported that glucose transport rate in skeletal muscle was higher when the muscles were perfused than when they were incubated. Comparative studies using both the perfusion procedure and incubated muscle preparations at the same time will be required to adequately explain the apparent differences between the studies. Second, in the present study, we used the glucose analog of 2-DG to measure glucose transport, while 3-O-methyl-D-glucose (3-MG) was used in the study by Etgen et al. The magnitude of the increase in glucose transport measured by using 2-DG in muscle from trained rats was similar to that measured by using 3-MG (21). Therefore, it is unlikely that the difference in the glucose analog used in the two studies can explain the differences.

The swimming-training protocol employed in the
present study induced a large increase in insulin responsiveness and GLUT-4 protein content 18 h after the training, and these training effects returned to control level 90 h after the training. However, at this time point after the training, citrate synthase activity remained at the level as high as that observed 18 h after cessation of training (Table 1). These results indicate that the increment in insulin responsiveness disappears much earlier than that of citrate synthase activity after the end of training. Previous studies (15, 19) reported that both GLUT-4 protein and citrate synthase activity in trained muscle returned to control levels after 7–10 days detraining. From these results, the authors suggested that muscle GLUT-4 protein content and oxidative enzyme activity undergo parallel alteration with detraining. However, because the half-life of citrate synthase as well as other mitochondrial oxidative enzymes in skeletal muscle is ~6–7 days (1), >50% of the increase in citrate synthase activity should be gone after 7–10 days of detraining. In the present investigation, both citrate synthase activity and GLUT-4 protein content were examined 18, 42, and 90 h after training. We found that citrate synthase activity remained at a higher level than sedentary control 90 h after training is stopped (Table 1). In contrast, GLUT-4 protein content had returned to the control level at the same time point after the training. These results indicate that GLUT-4 protein has a shorter half-life than does citrate synthase.

In conclusion, when the rats were trained by means of swimming for 2 h/day with a weight equal to 2% of body mass, insulin responsiveness and GLUT-4 protein content in epitrochlearis muscle were increased by ~85% 18 h after training. An increase in insulin responsiveness was still evident 42 h after the end of training and returned to sedentary control level within

### Table 1. Citrate synthase activities in epitrochlearis muscles of age-matched sedentary control and swimming rats after 5 days of swimming training

<table>
<thead>
<tr>
<th>Time After Swimming Training, h</th>
<th>18</th>
<th>42</th>
<th>66</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.4 ± 0.89*</td>
<td>24.1 ± 1.34*</td>
<td>24.3 ± 0.86</td>
<td>24.3 ± 1.46</td>
</tr>
<tr>
<td>(10)</td>
<td>(8)</td>
<td>(4)</td>
<td>(8)</td>
<td></td>
</tr>
<tr>
<td>Training</td>
<td>36.0 ± 1.25*</td>
<td>35.5 ± 1.03*</td>
<td>37.9 ± 0.87*</td>
<td>35.2 ± 1.62*</td>
</tr>
<tr>
<td>(13)</td>
<td>(7)</td>
<td>(8)</td>
<td>(8)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE for nos. of muscles in parentheses. Muscles for analyzing citrate synthase activities were excised 18, 42, 66, and 90 h after 5 days of swimming. *Significantly different from age matched control, P < 0.01.

For a given increase in GLUT-4 protein content within
the present study may partially explain the greater increase in insulin responsiveness on the next day after training.
90 h after training. The time course of change in insulin responsiveness after training paralleled the change in GLUT-4 protein content. These results suggest that the greater the increase in GLUT-4 protein content that is induced by training, the longer an effect on insulin responsiveness persists after the training is stopped.

The authors appreciate the provision of the antibody against GLUT-4 protein by Dr. Osamu Ezaki (National Institute of Health and Nutrition, Tokyo, Japan) and they also appreciate the stimulating discussion with Dr. Satoshi Shimegi (University of Osaka, Osaka, Japan). They thank Dr. John O. Holloszy (Washington University School of Medicine, St. Louis, MO) for the generous suggestions and help in editing the English of the manuscript.

This work was supported by research grants from the Meiji Life Foundation of Health and Welfare (Tokyo) (to K. Kawanaka), the Human Science Foundation (Tokyo) (to M. Higuchi), and Grant-in-Aid for Scientific Research (C) No. 08680158 from the Ministry of Human Science Foundation (Tokyo) (to M. Higuchi), and Grant-in-Fundation of Health and Welfare (Tokyo) (to K. Kawanaka), the School of Medicine, St. Louis, MO) for the generous suggestions and discussion with Dr. Satoshi Shimegi (University of Osaka, Osaka, Japan) and they also appreciate the stimulation of Dr. D.B. Elander (School of Medicine, Campus Box 8113, 660 S. Euclid Ave., St. Louis, MO 63110-1093).

Received 7 October 1996; accepted in final form 25 July 1997.

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