Decreased insulin-stimulated GLUT-4 translocation in glycogen-supercompensated muscles of exercised rats

KENTARO KAWANAKA, DONG-HO HAN, LORRAINE A. NOLTE, POLLY A. HANSEN, AKIRA NAKATANI, AND JOHN O. HOLLOSZY
Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

Kawanaka, Kentaro, Dong-Ho Han, Lorraine A. Nolte, Polly A. Hansen, Akira Nakatani, and John O. Holloszy. Decreased insulin-stimulated GLUT-4 translocation in glycogen-supercompensated muscles of exercised rats. Am. J. Physiol. 276 (Endocrinol. Metab. 39):E907–E912, 1999.—It was recently found that the effect of an exercise-induced increase in muscle GLUT-4 on insulin-stimulated glucose transport is masked by a decreased responsiveness to insulin in glycogen-supercompensated muscle. We evaluated the role of hexosamines in this decrease in insulin responsiveness and found that UDP-N-acetylhexosamine concentrations were not higher in glycogen-supercompensated muscles than in control muscles with a low glycogen content. We determined whether the smaller increase in glucose transport is due to translocation of fewer GLUT-4 to the cell surface with the 2-N-4(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis(o-mannose-4-ylxy)-2-propylamine (ATB-[2-3H]BMPA) photolabeling technique. The insulin-induced increase in GLUT-4 at the cell surface was no greater in glycogen-supercompensated exercised muscle than in muscles of sedentary controls and only 50% as great as in exercised muscles with a low glycogen content. We conclude that the decreased insulin responsiveness of glucose transport in glycogen-supercompensated muscle is not due to increased accumulation of hexosamine biosynthetic pathway end products and that the smaller increase in glucose transport is mediated by translocation of fewer GLUT-4 to the cell surface.

Exercise training; glucose transport; hexosamine; insulin responsiveness

The GLUT-4 isoform of the glucose transporter is translocated from intracellular sites to the cell surface in skeletal muscle in response to insulin and exercise (reviewed in Refs. 10, 17). This is the mechanism by which these stimuli increase muscle glucose transport (10, 17). There is a good correlation between the GLUT-4 content of a muscle and maximally stimulated glucose uptake (15, 23). Numerous studies have shown that exercise training induces an increase in skeletal muscle GLUT-4 content (2, 6, 9, 30, 31, 37), with a proportional increase in insulin-stimulated glucose transport (19, 22, 30, 31, 37).

There is evidence that large increases in muscle glycogen are associated with development of insulin resistance (3, 5, 16, 21, 34). Both the rate and extent of muscle glycogen supercompensation in response to carbohydrate feeding after glycogen-depleting exercise are markedly enhanced in rats that have adapted to exercise with an increase in muscle GLUT-4 (27). The term “glycogen supercompensation” refers to an increase in muscle glycogen concentration far above that found in the sedentary fed state. It was recently found that the effect of a training-induced increase in muscle GLUT-4 on insulin-stimulated glucose transport is masked by a decreased responsiveness to insulin that develops concomitantly with glycogen supercompensation (19).

Incubation or perfusion of muscles or adipocytes with glucosamine causes insulin resistance (1, 24, 35, 38). Studies on adipocytes have provided evidence suggesting that rapid uptake of large amounts of glucose causes insulin resistance that is mediated by entry of some of the glucose into the hexosamine biosynthetic pathway (24–26). The final products of the hexosamine pathway, UDP-N-acetylglucosamine (UDP-GlcNAc) and other UDP-hexosamines, are thought to be responsible for the insulin resistance. One purpose of this study was to determine whether there is an increase in hexosamine products in markedly glycogen-supercompensated muscles that could explain the decreased insulin responsiveness of glucose transport associated with high glycogen levels.

A decrease in insulin responsiveness, such as occurs in glycogen-supercompensated muscles, could be caused by either the translocation of fewer GLUT-4 to the cell surface or a reduction in the intrinsic glucose transport activity of the glucose transporters. The second purpose of this study was to distinguish between these possibilities by measuring the quantity of cell surface GLUT-4 in maximally insulin-stimulated muscles.

METHODS

Animal care and exercise programs. These studies were approved by the Animal Studies Committee of Washington University School of Medicine.

Muscle UDP-linked hexosamine accumulation study. The triceps muscles used for measurement of hexosamines were obtained from rats in our previous glycogen-supercompensation study, which has been described in detail (27). Briefly, 7-wk-old Wistar rats were exercised by means of a progressive swimming program and were swimming 6 h/day for the 3 wk preceding the glycogen supercompensation study. After an overnight fast, groups of trained and untrained rats were exercised with a muscle glycogen depletion swimming protocol (27). After the exercise, the untrained rats and one group of trained animals were provided with rat chow ad libitum for 24 or 48 h. A group of sedentary rats and a second group of trained exercised rats were studied after an overnight fast. Rats were anesthetized with pentobarbital sodium (5 mg/100 g body wt), and their triceps muscles were dissected out, clamp-frozen, and stored at −80°C until the hexosamine assay was performed.

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GLUT-4 translocation study. Wistar rats weighing ~115 g were assigned to exercise or control groups. Rats in the exercise group were accustomed to swimming for ~10 min/day for 2 days. They then were trained with a 2-day training protocol, described previously (19), that consists of swimming for 6 h in two, 3-h-long bouts separated by a 45-min long rest. After completion of the swimming on the second day, some of the rats were kept fasting and the others were fed rat chow ad libitum. The sedentary animals were fasted overnight. Approximately 18 h after the second exercise bout, which is long enough for the acute effect of exercise on insulin responsiveness to wear off (3), the animals were anesthetized with pentobarbital sodium (5 mg/100 g body wt) given intraperitoneally. The epitrochlearis and triceps muscles were dissected out.

Incubation of muscles with insulin. The epitrochlearis and triceps muscles were incubated with shaking for 60 min at 30°C in 2 ml of oxygenated Krebs-Henseleit buffer (KHB) containing 8 mM glucose, 32 mM mannitol, 0.1% RIA grade bovine serum albumin, in the presence or absence of 2 μM d-glucose. The concentration of insulin was well above that required to stimulate glucose transport maximally in this preparation. The flasks were continuously gassed with 95% O2-5% CO2 during the incubations. To remove glucose from the interstitial space, muscles were then washed for 10 min in KHB containing 40 mM mannitol, 0.1% BSA, and 2 μM insulin if it was present in the previous incubation.

Measurement of glucose transport activity. Glucose transport activity was measured with the glucose analog 3-0-methyl-d-glucose (3-MG) as described previously (42). After the wash, some of the muscles were incubated at 30°C for 10 min with shaking in 1 ml of KHB containing 8 mM 3-0-[3H]methyl-d-glucose (2 μCi/ml), 32 mM [14C]mannitol (0.2 μCi/ml), and 2 μM insulin if present in previous incubations. The 3-[3H]methyl-d-glucose and [14C]mannitol were from New England Nuclear.

Measurement of α-methylaminoisobutyrate transport. The nonmetabolizable amino acid analog α-methylaminoisobutyrate (MeAIB) was used to measure system A amino acid transport activity as described previously (11). Muscles were incubated in the presence or absence of 2 μM insulin exactly as described above for the measurement of glucose transport activity. After the wash step, muscles were incubated at 30°C for 20 min in 1.0 ml KHB containing 0.1 mM [14C]MeAIB (0.075 μCi/ml), 10 mM d-[2-3H]mannitol (0.375 μCi/ml), 0.1% BSA, and insulin if present in previous incubations. The [14C]MeAIB and d-[2-3H]mannitol were from New England Nuclear.

Photolabeling of epitrochlearis muscles. After the wash, some of the muscles were used for determination of the quantity of GLUT-4 at the cell surface with the use of the 2-N-4-(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis(o-mannose-4-yl-oxy)-2-propylamine (ATB-2-[3H]BMPA; Toronto Research Chemicals) exofacial photolabeling technique as described previously (13), except that the labeled GLUT-4 was precipitated with a rabbit polyclonal antibody followed by protein A-Sepharose.

Measurement of muscle GLUT-4 protein content. Muscle GLUT-4 glucose transporter content was determined by Western blotting with a rabbit polyclonal antibody directed against the COOH terminus of GLUT-4 as previously described (14).

Muscle glycogen. Percoll acid extracts of muscle were assayed for glycogen by the amyloglucosidase method (29).

Measurement of UDP-linked hexoses and hexosamines. Frozen muscles were homogenized in 4 vol (wt/vol) of 0.6 M perchloric acid. The homogenates were centrifuged at 2,000 g for 10 min. The PCA was extracted from the supernatant with 2 vol of 1:4 trioctylamine:1,1,2-trichlorotrifluoroethane (1:4 vol/vol). The aqueous phase was analyzed for UDP-GlcNAc, UDP-N-acetyl-galactosamine (UDP-GalNAC), and UDP-hexoses by high-performance liquid chromatography by the method of Holstge et al. (18). The UDP-hexoses and UDP-hexosamines were eluted at 22°C by flow and buffer gradients; peaks were quantified by ultraviolet absorption and comparison to standards.

Statistics. Results are expressed as means ± SE. The significance of differences among groups was evaluated with a one-way analysis of variance. Post hoc analyses were done with a Newman-Keuls test with significance level set at P < 0.05. Correlations were analyzed with univariate linear regression.

RESULTS

UDP-linked hexoses and hexosamines. Triceps muscle glycogen concentration in the glycogen-supercompensated muscles of the trained, exercised, and fed animals was 140 ± 12 μmol glucosyl/g wet wt and 51 ± 5 μmol/g in the glycogen-supercompensated muscles of the untrained, exercised, and fed rats 24 h after the depletion exercise, and glycogen did not increase further over the next 24 h. The average muscle glycogen concentration of fasted sedentary animals was 11.2 ± 1.0 μmol/g, whereas that of the fasted trained animals was 11.1 ± 1.5 μmol/g.

There were no differences between the values obtained on the muscles from the 24- and the 48-h postexercise fed animals in the UDP-linked hexoses or hexosamines; the 24- and 48-h values were, therefore, combined. As shown in Table 1, UDP-GlcNAc, UDP-GalNAC, and UDP-hexoses were not elevated in the massively glycogen-supercompensated muscles of the exercised fed animals compared with the muscles of sedentary fasted or trained, exercised, and fasted animals with low muscle glycogen levels. UDP-GlcNAc concentration was actually ~25% higher in the muscles of the fasted animals with a low glycogen concentration. There were also no significant differences in the concentrations of the UDP-linked hexoses and hexosamines between the muscles of the untrained, exercised, and fed rats and those of the trained, exercised, and fed rats (Table 1).

Glucose transport activity. Maximally insulin-stimulated glucose transport activity was measured in epitrochlearis muscles of sedentary fasted, 2-day exercised

Table 1. Concentrations of UDP-Hex, UDP-GlcNAc, and UDP-GalNAC in triceps muscle

<table>
<thead>
<tr>
<th></th>
<th>Untrained</th>
<th>Untrained</th>
<th>Trained</th>
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<tbody>
<tr>
<td></td>
<td>Fat</td>
<td>Exercised</td>
<td>Fat</td>
<td>Exercised</td>
<td>Fat</td>
</tr>
<tr>
<td>UDP-Hex</td>
<td>19.6 ± 1.0</td>
<td>22.5 ± 1.2</td>
<td>21.9 ± 1.2</td>
<td>21.3 ± 1.9</td>
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<tr>
<td>UDP-GlcNAc</td>
<td>16.2 ± 1.0</td>
<td>13.6 ± 0.9</td>
<td>17.6 ± 1.2</td>
<td>12.9 ± 0.7*</td>
<td></td>
</tr>
<tr>
<td>UDP-GalNAC</td>
<td>7.2 ± 0.3</td>
<td>8.2 ± 0.6</td>
<td>7.8 ± 0.2</td>
<td>7.9 ± 0.6</td>
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Values are means ± SE in nmol/g wet wt for 8 muscles/group except for trained + exercised + fasted group, in which means ± SE are for 3 muscles. UDP-Hex, UDP-hexoses; UDP-GlcNAc, UDP-N-acetylglucosamine; UDP-GalNAC, UDP-N-acetylgalactosamine. *P < 0.05, trained fed vs. untrained fasted rats.
fasted rats, and 2-day exercised fed rats ~18 h after the last exercise bout of the exercised animals. The muscles of exercised fed rats were glycogen supercompensated (Table 2). As shown in Fig. 1, the increase above basal in 3-MG transport induced by a maximally effective insulin stimulus was ~120% higher in the muscles of the exercise fasted rats than in those of the sedentary fasted animals. This enhancement of insulin-stimulated glucose transport activity was completely prevented in the glycogen-supercompensated muscles of the exercised fed rats. These results confirm those of our previous study (19).

Increase in GLUT-4 at the cell surface. A maximally effective insulin stimulus induced large increases in ATB-[2-3H]BMPA labeling of GLUT-4 (Fig. 2). The magnitude of this insulin-induced increase in cell surface GLUT-4 was 121% greater in muscles of the exercised fasted rats compared with the sedentary fasted rats. Thus the effect of exercise training on GLUT-4 translocation was very similar to the effect of exercise training on glucose transport activity. As shown in Fig. 2, the increase in GLUT-4 in the cell surface in response to insulin in the glycogen-supercompensated muscles of the fed exercised rats was no greater than in the fasted sedentary controls. The smaller increase in GLUT-4 at the cell surface explains the markedly lower, maximally insulin-stimulated glucose transport activity in the exercised muscles with a high glycogen content compared with those with a low glycogen content. Figure 3 shows the relationship between the increases above basal in 3-MG transport and cell surface GLUT-4 induced by insulin.

GLUT-4 protein. It was previously found that the 2-day swimming protocol used in this study induces a twofold increase in epitrochlearis muscle GLUT-4 (19, 31) and that the concentration of GLUT-4 is not affected by glycogen supercompensation (19). In the present study, we were unable to measure GLUT-4 in the epitrochlearis. However, we were able to confirm our previous findings with triceps muscle, which undergoes the same adaptation as the epitrochlearis (19). Triceps muscle GLUT-4 protein concentration was increased about twofold in the exercised groups (Table 2).

MeAIB transport. One mechanism by which the entry of large amounts of glucose into muscle, which leads to glycogen supercompensation, could cause insulin resistance is by inducing a decrease in expression and/or activation of one or more of the signaling proteins that

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Table 2. Concentrations of glycogen and GLUT-4 in muscle

<table>
<thead>
<tr>
<th></th>
<th>Sedentary Fasted</th>
<th>Exercised Fasted</th>
<th>Exercised Fed</th>
</tr>
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<tbody>
<tr>
<td>Glycogen, µmol/g tissue</td>
<td>11.1 ± 0.6 (6)</td>
<td>9.5 ± 1.0 (5)</td>
<td>81.3 ± 8.8* (5)</td>
</tr>
<tr>
<td>GLUT-4, U/µg protein</td>
<td>6.2 ± 1.0* (4)</td>
<td>13.7 ± 0.9 (7)</td>
<td>13.3 ± 1.0 (7)</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of muscles given in parentheses. Exercised animals performed swimming protocol described in Methods for 2 days and were studied 18 h after 2nd exercise session. *P < 0.05 vs. other 2 groups.

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Fig. 1. Maximally insulin-stimulated 3-O-methyl-D-glucose (3-MG) transport in epitrochlearis muscles of overnight fasted sedentary and exercised fasted rats and in glycogen-supersaturated muscles of exercised fed rats. Exercised rats performed 2 3-h-long bouts of swimming separated by a 45-min rest period on 2 successive days. One group of exercised rats was fasted, and other group was fed rat chow for 18 h before muscles were used for measurement of glucose transport. Values are means ± SE for 8–12 muscles/group. *P < 0.01, exercised fasted vs. sedentary fasted and exercised fed rats. †P < 0.05, exercised fasted vs. sedentary fasted rats.

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Fig. 2. Comparison of increases in cell surface GLUT-4 induced by 2 mU/ml of insulin in muscles of sedentary fasted, exercised fasted, and exercised fed rats. Muscles of fasted rats had low glycogen levels, whereas glycogen concentration was high in muscles of fed exercised rats. See legend of Fig. 1 for description of exercise protocol. Values are means ± SE for 3–5 muscles in basal groups and 5–6 muscles in insulin-treated groups. *P < 0.05, exercised fasted vs. sedentary fasted and exercised fed rats.

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Fig. 3. Relationship between increase above basal in cell surface GLUT-4 for 3 groups shown in Fig. 2 and increase above basal in 3-MG transport induced by a maximally effective insulin stimulus for 3 groups shown in Fig. 1.
mediate the effect of insulin. Stimulation of system A amino acid transport by insulin involves the same steps in the insulin-signaling pathway that are known to be involved in the activation of glucose transport (12, 39). In this context, we measured insulin-stimulated MeAIB transport in glycogen-supercompensated muscles of exercised rats as a preliminary approach to evaluating the possibility that insulin signaling is impaired. As shown in Fig. 4, the increase in MeAIB uptake in response to insulin was ~45% lower in muscles of the exercised fed group than in those of the exercised fasted group.

**DISCUSSION**

Exercise training rapidly induces an increase in GLUT-4 protein in skeletal muscle (17). In the absence of insulin resistance, maximally insulin-stimulated glucose transport, i.e., insulin responsiveness, increases in proportion to the increase in GLUT-4 induced in muscles by exercise (19, 22, 30, 31, 37) and other stimuli (32, 41). The results of the present study (Fig. 2) and those reported by Reynolds et al. (33) show that the greater increase in glucose transport in response to insulin in trained than in untrained muscle is mediated by the translocation of more GLUT-4 to the cell surface. Our results further show that, in muscles with a low glycogen content, the increase in the quantity of GLUT-4 translocated into the plasma membrane by the action of insulin is proportional to the adaptive increase in muscle GLUT-4 induced by exercise. This relationship explains the proportionality between the increases in total GLUT-4 and insulin-stimulated glucose transport.

After glycogen-depleting exercise, muscle glucose transport activity is enhanced as a result of direct stimulation of glucose transport by exercise and increased insulin responsiveness. As these acute effects of exercise wear off, they are replaced by a large increase in insulin sensitivity (3, 7, 40). As a result of the increase in glucose transport activity, glucose floods into the muscles, leading to glycogen supercompensation if sufficient glucose is made available. This glycogen supercompensation phenomenon is markedly enhanced in muscles that have adapted to exercise with an increase in GLUT-4 (19, 27). An increase in glycogen synthase does not seem to play a role in this greater glycogen supercompensation, which appears to be mediated by more rapid glucose uptake by the muscles (19, 27, 31).

In a recent study, we found that glycogen supercompensation is associated with a decrease in insulin responsiveness of glucose transport that is so severe that it masks the effect of an exercise-induced, twofold increase in muscle GLUT-4 on the insulin responsiveness of glucose transport (19). The results of our cell surface GLUT-4 photolabeling experiment in the present study show that the smaller increase in glucose transport in response to insulin in glycogen-supercompensated muscle is explained by translocation of fewer GLUT-4 to the cell surface (Fig. 2). Because the decrease in GLUT-4 translocation is of the same magnitude as, and completely accounts for, the reduction in insulin-stimulated glucose transport, it is clear that a decrease in intrinsic activity of the glucose transporters does not play a role in the decreased insulin responsiveness of glucose transport associated with glycogen supercompensation.

It is well documented that perfusion or incubation of muscle or adipocytes with glucosamine causes insulin resistance (1, 24, 35, 38). It has been hypothesized, on the basis of studies on adipocytes, that the insulin resistance caused by rapid entry of large amounts of glucose into insulin-responsive tissues, i.e., “glucose toxicity,” is mediated by increased glucose entry into the hexosamine biosynthetic pathway with accumulation of UDP-N-acetyl hexosamines (24–26). In this context, it seemed probable to us that rapid entry of glucose into muscle after glycogen-depleting exercise results in accumulation of UDP-N-acetyl hexosamines with development of insulin resistance. Our finding that UDP-N-acetyl hexosamine concentrations are not elevated in the massively glycogen-supercompensated muscles of exercise-trained rats 24 and 48 h after glycogen-depleting exercise was, therefore, unexpected.

However, in retrospect, the experimental evidence that glucose toxicity insulin resistance is mediated by accumulation of hexosamine pathway end products in skeletal muscle is not very convincing (28, 36). In the study by Nelson et al. (28), in which muscles were studied immediately after exercise and 16 h after exercise, the concentration of UDP-HexNAc was actually lower in muscles of fed rats 16 h after exercise than in muscles taken immediately after exercise. In the study of Robinson et al. (36), an extreme degree of hyperglycemia was associated with only a 15% increase in muscle UDP-HexNAc. Furthermore, the recent finding by Hresko et al. (20) that the insulin resistance caused by exposure of adipocytes to glucosamine may be due to an artifact rather than a physiological phenomenon suggests that the hexosamine hypothesis of insulin resistance may need reevaluation. Hresko et al. found that incubation of adipocytes with glucosamine causes severe depletion of ATP.

Another explanation for the decreased insulin responsiveness associated with glycogen supercompensation...
is suggested by a report by Coderre et al. (4) that a large portion of the GLUT-4-containing vesicles in skeletal muscle are bound to glycogen. If this is correct, binding of progressively more GLUT-4 to glycogen as glycogen supercompensation occurs would result in a decrease in the number of GLUT-4 vesicles available for translocation in response to insulin. This is an attractive concept that we are in the process of trying to substantiate, so far without success.

Rapid entry of glucose into hepatocytes and adipocytes induces adaptive changes in the expression of a variety of enzymes (8). This raises the alternative possibility that in muscle undergoing glycogen supercompensation, a metabolite of glucose might mediate a change in the expression and/or activity of one or more of the insulin-signaling proteins involved in the stimulation of glucose transport. Our finding that insulin-stimulated MeAIB transport was also decreased in muscles of the exercised rats fed carbohydrate is compatible with this possibility, because the insulin-signaling steps involved in the stimulation of glucose transport also mediate activation of system A amino acid transport (12, 39). Clearly, this is an important area for future research.

In conclusion, the results of this study confirm that glycogen supercompensation is associated with development of a large decrease in insulin responsiveness of muscle glucose transport. This decrease in insulin responsiveness is so marked that it completely masks the effect of an exercise-induced twofold increase in muscle GLUT-4. Our results show that the smaller increase in glucose transport in response to insulin in glycogen-supercompensated muscle is explained by translocation of fewer GLUT-4 to the cell surface. There was no increase above control in UDP-N-acetyl hexosamines in glycogen-supercompensated muscles, providing evidence that the decreased insulin responsiveness is not due to accumulation of hexosamine pathway end products.

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Address for reprint requests and other correspondence: J. O. Holloszy, Washington Univ. School of Medicine, Division of Geriatrics and Gerontology, 4566 Scott Ave., Campus Box 8113, St. Louis, MO 63110 (E-mail: jhollosz@mgate.wustl.edu).

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