How muscle insulin sensitivity is regulated: testing of a hypothesis

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Geiger, Paige C., Dong Ho Han, David C. Wright, and John O. Holloszy. How muscle insulin sensitivity is regulated: testing of a hypothesis. Am J Physiol Endocrinol Metab 291: E1258–E1263, 2006.—Muscle contractions induce an increase in glucose transport. The acute effect of muscle contractions on glucose transport is independent of insulin and reverses rapidly after cessation of exercise. As the acute increase in glucose transport reverses, a marked increase in the sensitivity of muscle to insulin occurs. The mechanism for this phenomenon is unknown. We hypothesize that an increase in insulin sensitivity is a general phenomenon that occurs during reversal of an increase in cell surface GLUT4 induced by any stimulus, not just exercise. To test this hypothesis, epitrochlearis, rat soleus, and flexor digitorum brevis muscles were incubated for 30 min with a maximally effective insulin concentration (1.0 mU/ml). Muscles were then exposed to 60 μU/ml insulin for 30 min followed by measurement of glucose transport. Preincubation with 1.0 μU/ml insulin resulted in an ~2-fold greater increase in glucose transport 3.5 h later in response to 60 μU/ml insulin than that which occurred in control muscles treated with 60 μU/ml insulin. Pretreatment of muscles with combined maximal insulin and exercise stimuli greatly amplified the increase in insulin sensitivity. The increases in glucose transport were paralleled by increases in cell surface GLUT4. We conclude that stimulation of glucose transport by any agent is followed by an increase in sensitivity of glucose transport to activation that is mediated by translocation of more GLUT4 to the cell surface.

hypothesis; exercise; glucose transport; glucose transporter

GLUCOSE TRANSPORT can be stimulated by at least two independent pathways in skeletal muscle. The insulin-signaling pathway is activated when insulin binds to its receptor (25). Another pathway is activated by exercise/contractions and hypoxia (2, 21, 22). As the acute effect of muscle contractions on glucose transport reverses, it is replaced by a large increase in insulin sensitivity (3, 10, 36). Insulin sensitivity is defined in terms of the insulin concentration that induces 50% of its maximal effect (24). Thus, when insulin sensitivity is increased, a given submaximal insulin stimulus results in a larger increase in glucose transport, with no increase in maximally insulin-stimulated transport. This phenomenon does not appear to be mediated by augmentation of the insulin signal (6, 12, 16, 37, 41, 42). There is evidence that the increase in insulin sensitivity is mediated by translocation of a greater number of the glucose transporter 4 (GLUT4) isoform of the glucose transporter to the cell surface in response to a given submaximal insulin stimulus (16). Although it is referred to as an increase in insulin sensitivity, this phenomenon involves an increase in sensitivity not only to insulin but also to other agents that stimulate muscle glucose transport, such as hypoxia (6).

Both insulin and contractions induce graded increases in glucose transport, with a progressive augmentation of glucose transport activity when the magnitude of the stimulus is increased, until a maximal effect is attained. In unstimulated muscles, most of the GLUT4 are intracellular (5, 26, 39). Stimulation of glucose transport by insulin is mediated by translocation of GLUT4 from intracellular sites to the cell surface (4, 29–31, 33, 34, 40). In the case of skeletal muscle, the bulk of the GLUT4 is translocated to the transverse tubules, with a smaller amount moving to the sarcolemma (31, 35, 39). A possible explanation for the graded response is that stronger insulin signals are generated in some regions of the muscle cell than in others. GLUT4 that are translocated in response to a low concentration of insulin are in regions where the strongest insulin signals are generated. In regions of the cell where the insulin signal is more attenuated, GLUT4 are translocated when insulin concentration is raised sufficiently to increase the strength of signal to the threshold at which GLUT4 translocation occurs. In support of this possibility, treatment of muscle with a peroxovanadate compound that activates the insulin-signaling pathway much more powerfully than does a maximal insulin stimulus results in ~2-fold greater increases in GLUT4 translocation and glucose transport than a maximal insulin stimulus (33). The effect of peroxovanadate on glucose transport is completely blocked by wortmannin, showing that it is mediated entirely by the insulin-signaling pathway and not by activating other pathways.

In this context, it seemed possible that increased insulin sensitivity could be mediated by movement of GLUT4 transporters into regions where they are more susceptible to recruitment to the cell surface by a weak signal. In the case of the exercise-induced increase in insulin sensitivity, this could occur as the result of movement of GLUT4 into “high susceptibility” regions when they leave the cell surface, since they undergo endocytosis during reversal of the increase in glucose transport. Once the stimulus that induced GLUT4 translocation is removed, it would seem that, during the process of GLUT4 endocytosis, the nature of the stimulus that brought the GLUT4 to the cell surface is irrelevant. Therefore, if this explanation is correct, it appears reasonable that stimulation of glucose transport by insulin should also be followed by an increase in insulin sensitivity. The purpose of the present study was to test this possibility.

METHODS

Materials. 2-Deoxy-[1,2-3H]glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). [14C]mannitol was
obtained from ICN Radiochemicals (Irvine, CA). Anti-phospho (Thr\textsuperscript{140}/Thr\textsuperscript{142})-p38 MAPK and total p38 MAPK antibodies were purchased from Cell Signaling (Beverly, MA), and anti-phospho (Thr\textsuperscript{180}/Thr\textsuperscript{182})-Akt/PKB\textalpha, anti-phosphatidylinositol (PI) 3-kinase, and anti- tiphosphoryserine antibodies were obtained from Upstate (Lake Placid, NY). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). All other chemicals were obtained from Sigma (St. Louis, MO).

Treatment of rats and muscle preparations. Male Wistar rats (Charles River) weighing ~80–120 g were provided with Purina Rat Chow and water ad libitum. On the evening before the experiment, food was removed. For the exercise studies, one group of rats was exercised by means of swimming for 2 h, as described previously (3). An intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) was used to anesthetize the rats, followed by the removal of the epitrochlearis (EPI), soleus, and flexor digitorum brevis (FDB) muscles (18). The EPI, a small, thin muscle of the forelimb, is well suited for studies of glucose transport. It contains predominantly type IIb (fast-twitch white) fibers. Soleus muscles, which consist predominantly of type I (slow-twitch red) fibers, were split longitudinally into strips prior to incubation, as described previously (19), to allow adequate diffusion of oxygen and substrates. The FDB contains predominantly type Ila (fast-twitch red) fibers (1). All protocols were approved by the Animal Studies Committee of Washington University.

Muscle treatments. After dissection, muscles recovered for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol (recovery medium), and a gas phase of 95% O\textsubscript{2}-5% CO\textsubscript{2}. The muscles were placed in a shaking incubator maintained at 35°C. After recovery, muscles were incubated in rat serum in the presence or absence of 1.0 mU/ml insulin for 30 min. The muscles were then allowed to recover for 3 h in the recovery medium, followed by 30 min of incubation in the same medium with or without 60 μM insulin or with or without 1 mM insulin. These time intervals were the same as those used for studies of the effects of exercise, in vitro contractions, hypoxia, and 5-aminomimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (6, 7, 16). Some of these experiments also included insulin treatment.

Measurement of glucose transport activity. The muscles were rinsed for 10 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol and insulin, if it was present during the previous incubation. After the rinse step, muscles were incubated for 20 min at 29°C in flasks containing 2 ml of KHB with 4 mM 2-deoxy-[\textsuperscript{1,2-}\textsuperscript{3}H]glucose (2-DG; 1.5 μCi/ml) and 36 mM [\textsuperscript{14}C]mannitol (0.2 μCi/ml), with a gas phase of 95% O\textsubscript{2}-5% CO\textsubscript{2} in a shaking incubator (15). The same additions that were in the rinse were present during the determination of glucose transport. The muscles were then blotted and clamp-frozen and processed for determination of intracellular 2DG accumulation and extracellular space, as described previously (43).

Measurement of insulin signaling and p38 MAPK phosphorylation. Preparation of muscle extracts, immunoprecipitation, and quantification of phosphotyrosine-bound PI 3-kinase, and phosphorylation of PKB on Thr\textsuperscript{308} and Thr\textsuperscript{473}, p38 MAPK, and phospho-p38 MAPK by immunoblotting were performed as described previously (11, 27, 34).

Photolabeling of muscle cell surface GLUT4. Epitrochlearis muscles were treated as described above, but instead of measurement of 2-DG transport, the muscles were incubated in KHB containing 0.5 mM biotinylated 2-N\textsubscript{2}-(3-aza-2,2,6,6-tetramethylpiperidin-4-yloxy)-2-propylamine (ATB-BMPA) for 8 min at 18°C in the dark. Muscles were then irradiated for 2 min with a mercury vapor lamp (450 W; Conrad Hanovia); the muscles were then turned, and the other side was radiated for 2 min. Isolation of biotinylated GLUT4 was performed as described by Hashimoto et al. (17). After irradiation, the muscles were homogenized in 255 mM sucrose containing 1 mM EDTA, 20 mM HEPES, 1 μg/ml each of antipain, aprotinin, pepstatin, and leupeptin, and 100 μM 4-(2-aminophenoxy)benzenesulfonyl fluoride hydrochloride (AEBSF). The homogenates were centrifuged at 165,000 g at 4°C for 90 min to obtain a membrane fraction. The pellet was solubilized in Thesit detergent buffer containing (wt/g) 2% Thesit, 5 mM sodium phosphate, and 5 mM EDTA, pH 7.2, and 1 μg/ml each of antipain, aprotinin, pepstatin, and leupeptin and 100 μM AEBSF. The pellets were solubilized for 60 min with rotation at 4°C, followed by centrifugation at 20,000 g for 20 min at 4°C. The supernatants were treated with streptavidin beads (Pierce, Rockford, IL) to capture biotinylated proteins. The streptavidin precipitates were washed four times with PBS containing 0.1% Thesit and protease inhibitors and once with PBS. Laemmli electrophoresis sample buffer was added to each pellet, and the samples were heated at 95°C for 30 min, followed by centrifugation at 2,300 g for 1 min. This procedure was repeated, and the supernatants were combined. Mercaptoethanol was added to the supernatants to give a final concentration of 10%. The samples were then subjected to SDS-PAGE for measurement of GLUT4 protein (9).

Statistical analysis. Data are presented as means ± SE. Comparisons between the means of multiple groups were made using a one-way ANOVA followed by a post hoc comparison using Fishers least significant difference method. Statistical significance was set at P < 0.05.

RESULTS

Insulin treatment is followed by an increase in insulin sensitivity. The glucose transport process is usually characterized in terms of its sensitivity and responsiveness to insulin (24). Insulin sensitivity is defined in relation to the concentration of insulin required to induce 50% of its maximal effect on glucose transport, whereas insulin responsiveness is defined as the increase in glucose transport induced by a maximally effective insulin concentration. In our rat EPI muscle preparation, 1.0 mU insulin stimulates glucose transport maximally, whereas 60 μU/ml insulin results in ~50% of the maximal effect (38). As shown in Fig. 1A, exposure of EPI muscles to 1.0 mM insulin for 30 min was followed 3 h later by an ~2-fold amplification of the response of glucose transport to treatment with 60 μU/ml insulin for 30 min. Similar increases in insulin sensitivity occurred in the soleus and flexor digitorum muscles (data not shown). The magnitude of this increase in insulin sensitivity is evident from a comparison of the increases in 2DG transport induced by 60 μU/ml insulin in the control and insulin-pretreated muscles (Fig. 1A).

To determine whether the increase in insulin sensitivity of glucose transport induced by pretreatment with a maximal insulin stimulus is mediated by translocation of more GLUT4 to the cell surface, we measured GLUT4 at the cell surface using a biotinylated ATB-BMPA photolabel. As shown in Fig. 1B, pretreatment with 1.0 mM/ml insulin resulted in a marked increase in the amount of GLUT4 brought to the cell surface by 60 μU/ml insulin. The increase in insulin sensitivity of glucose transport induced by pretreatment with a maximal insulin stimulus is completely accounted for by the greater increase in cell surface GLUT4.

Effects of swimming and insulin pretreatment on insulin sensitivity are additive. The maximal effects of insulin and contractions on muscle glucose transport and GLUT4 translocation are additive (18, 30, 32, 33, 38, 44). In the present study, we evaluated whether the combination of swimming and insulin treatment results in a greater increase in insulin sensitivity than with either treatment alone. A 2-h swim followed by treatment

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of EPI muscles with 1.0 mU/ml insulin for 30 min and 3 h of recovery resulted in an increase in sensitivity of glucose transport to 60 μU/ml insulin that is greater than that induced by pretreatment with insulin alone (Fig. 2A). Interestingly, after combined swimming and insulin pretreatment, the increase in glucose transport induced by 60 μU/ml insulin 3 h after insulin pretreatment was as great as the response to a maximally effective insulin concentration (Fig. 3A). Thus, as a result of the increase in insulin sensitivity induced by pretreatment with insulin, a concentration of insulin that usually has one-half of the maximal effect of insulin on glucose transport had a maximal insulin effect.

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As shown in Fig. 2B, the combined effects of swimming and insulin pretreatment also resulted in a 2-fold greater increase in the amount of GLUT4 brought to the cell surface by 60 μU/ml insulin than did insulin pretreatment alone.

Insulin pretreatment is not followed by an increase in muscle insulin responsiveness. To determine whether insulin pretreatment results in an increase in the responsiveness of muscle glucose transport to insulin, we incubated muscles with a maximally effective insulin concentration (1 mU/ml) after 3 h of recovery from the initial insulin stimulus. As shown in Fig. 3A, the response of glucose transport to 1 mU/ml insulin 3 h after insulin pretreatment was no greater than the acute response to the same insulin stimulus. This finding demonstrates that insulin pretreatment does not increase insulin responsiveness of muscle glucose transport. Interestingly, the increase in glucose transport induced by 60 μU/ml insulin 3 h after insulin pretreatment was as great as the response to a maximally effective insulin concentration (Fig. 3A). Thus, as a result of the increase in insulin sensitivity induced by pretreatment with insulin, a concentration of insulin that usually has one-half of the maximal effect of insulin on glucose transport had a maximal insulin effect.

Fig. 1. Insulin treatment is followed by increases in insulin sensitivity of glucose transport and glucose transporter 4 (GLUT4) translocation. Epitrochlearis muscles were treated with 1.0 mU/ml insulin for 30 min, allowed to recover for 3 h in the absence of insulin, and then incubated for 30 min in the presence of 60 μU/ml insulin. Control muscles were incubated for 3.5 h in the absence of insulin. 2-Deoxyglucose transport (A) or cell surface GLUT4 labeling with biotinylated 2-N-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(ω-mannos-4-ylxyl)-2-propylamine (ATB-BMPA) photolabel (B) was then measured. Values are means ± SE for 8–10 muscles for 2-deoxyglucose transport and 6–8 muscles/group for cell surface GLUT4 labeling. *P < 0.001 vs. no insulin pretreatment control.

Fig. 2. Swimming plus insulin pretreatment is followed by a greater increase in insulin sensitivity than is induced by insulin pretreatment alone. Epitrochlearis muscles from rats that had swum for 2 h and from sedentary control rats were incubated with or without 1.0 mU/ml insulin for 30 min, followed by 3 h of incubation in the absence of insulin. Muscles were then incubated for 30 min in the absence or presence of 60 μU/ml insulin, followed by measurement of 2-deoxyglucose transport (A) or cell surface GLUT4 with biotinylated ATB-BMPA photolabel (B). Values are means ± SE for 8–10 muscles/group for 2-deoxyglucose transport and 6–8 muscles/group for cell surface GLUT4 labeling. *P < 0.001 vs. no insulin pretreatment control; †P < 0.001 vs. insulin pretreatment group.
As shown in Fig. 3B, pretreatment with 1.0 mU/ml insulin also had no effect on the insulin responsiveness of GLUT4 translocation to the cell surface.

**Insulin signaling.** The increase in insulin sensitivity induced by exercise does not appear to be mediated by amplification of the insulin signal. However, this does not rule out the possibility that the increase in insulin sensitivity induced by pretreatment with insulin is mediated by an augmentation of the insulin signal. To evaluate this possibility, we determined the effect of pretreatment with 1 mU/ml insulin on the extent to which PI 3-kinase is bound to phosphotyrosine and PKB phosphorylation on Thr308 and Thr473 in response to 60 μU/ml insulin. As shown in the representative Western blots in Fig. 4, 60 μU/ml insulin caused similar (i.e., not statistically significantly different) increases in phosphotyrosine-bound PI 3-kinase and PKB phosphorylation on Thr308 and Thr473 in the control muscles and muscles pretreated with 1 mU/ml insulin (i.e., no amplification of insulin signaling). We (11) recently found that phosphorylation of p38 MAPK in response to anisomycin is followed by an increase in muscle insulin sensitivity. Therefore, we evaluated the effect of insulin pretreatment on p38 phosphorylation. There was no augmentation of the small increase in p38 phosphorylation in response to 60 μU/ml insulin in the muscles pretreated with 1 mU/ml insulin (Fig. 4).

**DISCUSSION**

This study provides the new information that treatment of muscles with a maximally effective insulin stimulus is followed by an increase in sensitivity of glucose transport to stimulation by insulin and that this effect is mediated by a greater increase in GLUT4 at the cell surface. This response appears to be directly comparable with the increase in insulin sensitivity that develops in muscle after exercise/contractions, activation of AMP-activated protein kinase with AICAR, or hypoxia (20). The present results show that the increase in sensitivity is not a specific response to exercise (3, 10, 36) and exercise mimetics (6) but also occurs after stimulation of glucose transport via the insulin-signaling pathway.

Both insulin-stimulated and contraction/hypoxia-stimulated glucose transport are mediated by translocation of the GLUT4.
isoform of the glucose transporter to the cell surface in skeletal muscle (4, 20, 23, 29, 30). In unstimulated muscle, most of the GLUT4 are located in sites within muscle cells (5, 26, 31, 39). The signals generated by insulin and by exercise/hypoxia cause GLUT4 to move to the surface of the sarcolemma. Normally, glucose transport activity is proportional to the number of active GLUT4 at the muscle cell surface (28–30, 33).

A phenomenon that has not been given much consideration in the development of models of GLUT4 trafficking and the regulation of glucose transport is the graded response that occurs in response to insulin and to contractions/hypoxia. For example, in our epitrochlearis muscle preparation the magnitude of the increase in glucose transport varies over an ~5-fold range, when insulin concentration is increased from 20 μU/ml to 1.0 mU/ml (38). A recent study by Govers et al. (13), where intracellular GLUT4 trafficking in 3T3-L1 adipocytes was analyzed using GLUT4 with a hemagglutinin tag, is of interest relative to this phenomenon. They obtained evidence that insulin releases GLUT4 from intracellular compartments into a cell surface recycling pathway in a graded, insulin concentration-dependent manner and that the size of the recycling pool of transporters progressively increased with increasing concentrations of insulin.

It was our working hypothesis that the insulin signals generated are stronger in some regions of the cell than in others and that the GLUT4 that are translocated in response to a low insulin concentration are located in regions where the insulin signal is strong. In regions of the cell where the insulin signal is more attenuated, recruitment of GLUT4 occurs when insulin concentration is raised sufficiently to increase the strength of the insulin signal to the threshold at which GLUT4 translocation occurs. If this scenario is correct, an increase in insulin sensitivity could occur as a result of movement of a greater proportion of the GLUT4 that are available for translocation into the region(s) where the insulin signal is strongest.

In this context we hypothesized that, during the recovery period after exercise/contractions, as they undergo endocytosis, the GLUT4 move from the cell surface into the regions of the cell where the strongest insulin signal is generated. As long as more GLUT4 remain in regions accessible to a weak insulin stimulus (i.e., where a strong signal is generated), insulin sensitivity would be increased. Methodology for testing this hypothesis directly in skeletal muscle is not available to us. Therefore, we examined it indirectly by determining whether the seemingly unlikely outcomes predicted by the hypothesis do in fact occur. Once the stimulus that induced GLUT4 translocation is removed, the nature of the stimulus that brought the GLUT4 to the cell surface becomes irrelevant during the process of GLUT4 endocytosis. Therefore, if our hypothesis is correct, any stimulus that brings GLUT4 to the cell surface should be followed by an increase in insulin sensitivity because of movement of the GLUT4 into regions where they are more susceptible to a weak insulin signal. This prediction proved correct, as evidenced by our finding that insulin sensitivity of muscle glucose transport is increased after recovery from an insulin stimulus.

If this general concept is correct, then the greater the number of GLUT4 translocated to the cell surface, the larger should be the number of GLUT4 that subsequently, during endocytosis, move into regions where they are highly susceptible to recruitment by a weak signal. The maximal effects of insulin and contractions on muscle glucose transport and GLUT4 translocation to the cell surface are additive (8, 18, 30, 32, 33, 38, 44). Therefore, if our hypothesis is correct, endocytosis into regions of high susceptibility to a weak signal of the larger number of GLUT4 brought to the cell surface by exercise plus insulin should result in a greater increase in insulin sensitivity than is induced by exercise or insulin alone. This prediction also proved to be correct, as evidenced by the finding that 60 μU/ml insulin results in ~2-fold greater increases in muscle glucose transport and cell surface GLUT4 after combined maximal stimulation with exercise plus insulin than in muscles that have recovered from a maximal insulin stimulus alone.

An alternative scenario that is also compatible with our results is that more of a protein that senses the insulin signal, or increases sensitivity to insulin, is incorporated into the GLUT4 vesicles that form to contain the GLUT4 as they move from the sarcolemma back into the cell. If the vesicles that contain GLUT4 that have recently been endocytosed contain more insulin sensing or sensitizing protein than GLUT4 vesicles that formed less recently, this could also explain the increase in insulin sensitivity. Our results do not distinguish between these possibilities.

It has been suggested that the increase in insulin sensitivity induced by exercise is due to “activation” of the glucose transporters at the cell surface and that the increase in insulin sensitivity is due to increased transport activity of the same number of GLUT4 rather than to translocation of more GLUT4 to the cell surface. If this was correct, the increased binding of photolabel to cell surface GLUT4 observed in muscles where insulin sensitivity is increased (Fig. 1) (16) would be explained by a greater affinity for and binding of the ATB-BMPA GLUT4 exofacial label to the same number of GLUT4, rather than to translocation of more GLUT4 to the cell surface. This hypothesis appears to be disproved by the finding that responsiveness of glucose transport to a maximally effective insulin stimulus is not increased either after exercise (14, 16) or after insulin treatment (Fig. 4). If the ~2-fold increase in insulin sensitivity was mediated by an ~2-fold increase in glucose transport activity of GLUT4, rather than by an increase in cell surface glucose transporters, there should be a proportional increase in insulin responsiveness as a result of the larger number of GLUT4 brought to the cell surface by a maximal insulin stimulus.

In conclusion, the present results show that, like exercise, AICAR, and hypoxia, a maximal insulin stimulus is followed by a large increase in muscle insulin sensitivity. Furthermore, the effects of exercise and insulin on insulin sensitivity are additive. Although these findings are compatible with and support the hypothesis that led to this study, there are other possible explanations, and new methods are needed to directly investigate this phenomenon.

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REGULATION OF MUSCLE INSULIN SENSITIVITY

GRANTS

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