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Exercise modulates the insulin-induced translocation of glucose transporters in rat skeletal muscle

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Insulin and acute exercise (45 min of treadmill run) increased glucose uptake into perfused rat hindlimbs 5-fold and 3.2-fold, respectively. Following exercise, insulin treatment resulted in a further increase in glucose uptake. The subcellular distribution of the muscle glucose transporters GLUT-1 and GLUT-4 was determined in plasma membranes and intracellular membranes. Neither exercise nor exercise→insulin treatment altered the distribution of GLUT-1 transporters in these medmbrane fractions. In contrast, exercise, insulin and exercise→insulin treatment caused comparable increases in GLUT-4 transporters in the plasma membrane. The results suggest that exercise might limit insulin-induced GLUT-4 recruitment and that following exercise, insulin may alter the intrinsic activity of plasma membrane glucose transporters.

Glucose uptake; Insulin action

1. INTRODUCTION

Skeletal muscle is the primary tissue responsible for insulin-dependent whole-body glucose uptake [1]. Like insulin, acute exercise increases glucose uptake in perfused hindlimbs and isolated muscles [2,3]. The effect of exercise persists for several hours after cessation of activity [2,3] and can be reproduced by electrical stimulation in vitro [4–6]. Although the underlying mechanism of enhanced glucose uptake is unclear, earlier studies showed that a combined exercise⟶insulin treatment had an additive effect on glucose uptake in skeletal muscles, suggesting that the exercise- and insulin-mediated stimulations of glucose uptake occur via independent pathways [3,6].

Recent studies have indicated that there are several subtypes of glucose transporters [7–13]. The human erythrocyte/Hep G2/rat brain glucose transporter (GLUT-1) [7,8] and the 'insulin regulatable glucose transporter' (GLUT-4) [9,10] are expressed in adult skeletal muscle. D-Glucose-protectable cytochalasin B binding is a measure of glucose transporter number irrespective of specific isoform [10,14]. Cytochalasin B binding studies have shown that insulin stimulation of glucose uptake in skeletal muscle involves recruitment of glucose transporters from an intracellular membrane fraction to the plasma membrane [15–17]. Similarly, it was recently reported that exercise also increases the number of cytochalasin B binding sites in rat hindlimb muscle plasma membrane fractions [17–19]. However in contrast to insulin, exercise did not cause a concomitant decrease in cytochalasin B binding sites in an insulin-sensitive intracellular membrane fraction [19]. The latter study therefore suggested the existence of distinct insulin- and exercise-recruitable transporter pools.

Here we studied the effect of combined exercise and insulin action (exercise⟶insulin) in an attempt to investigate whether separate pools of transporters mediate transport stimulation by each stimulus. Surprisingly, we found that exercise changes the mechanism of action of insulin, and we propose that there is a finite number of glucose transporters that can be recruited to the plasma membrane irrespective of the nature of the stimulus.

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2. MATERIALS AND METHODS

2.1. *Reagents*

D- and L-Glucose, cytochalasins B and E, and gradient grade sucrose were obtained from Sigma Chemical. Porcine insulin (Regular Iletin) was obtained from Eli Lilly. [^{3}H]Cytochalasin B was from Amersham. R 820 is a polyclonal antibody raised in rabbits to a synthetic peptide of a 12 amino acid C-terminus sequence of the GLUT-4 transporter, and was a kind gift from Dr David E. James, Washington University, St. Louis, MO. α G6 is a polyclonal antibody raised in rabbits to a synthetic peptide of a 15 amino acid C-terminus sequence of the GLUT-1 transporter, and was a kind gift from Dr Robert J. Smith, Joslin Diabetes Center, Boston, MA.

2.2. *Animals, exercise program, and perfused hindlimb preparation*

Male Sprague–Dawley rats weighing ~350 g were familiarized with treadmill running for 5 min/day. Rats were fasted at 17.00 h the

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night before the final 45 min exercise bout of treadmill running up a 15% grade, beginning at 20 m/min for 5 min followed by 40 min at 30 m/min. The rats were immediately anesthetized with sodium pentobarbital (60 mg/kg body wt), and surgically prepared for hindquarter perfusion as described earlier [15,19], with a 20 min long flow-through perfusion (20 ml/min) of Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 4 g/100 ml of bovine serum albumin. For combined exercise⟶insulin treatment, the muscles were perfused with insulin (1000 μU/ml) immediately following exercise. After perfusion, the hindlimb muscles were dissected out, trimmed of fat and connective tissue on ice and frozen in liquid N_2. Muscles were stored at −70°C until used for membrane preparation.

2.3. *Membrane preparation and characterization*

The protocol used for the isolation of plasma membranes and intracellular membranes, and the complete marker enzyme analysis of these membrane fractions have been previously described [15,19,20]. Plasma membranes obtained by this procedure showed >3-fold enrichment of the plasma membrane marker enzymes 5′-nucleotidase, (Mg^{2+})-ATPase and phosphodiesterase I relative to the crude (total) membranes. This represents a >15-fold enrichment relative to muscle homogenates [21]. In contrast, the intracellular membrane fractions were not enriched in these marker enzymes. Protein was measured by the method of Lowry et al. [22]. The total protein yield in control plasma membrane and intracellular membrane fractions were 1.45 ± 0.09 and 10.3 ± 0.93 mg protein (n = 6), respectively (starting material was 32.66 ± 1.95 g, n = 6). Neither exercise nor exercise⟶insulin treatment altered the protein recoveries or purity of the membrane fractions.

2.4. *Western blot analysis*

Membranes (30 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels according to Laemmli [23] and electrophoretically transferred to nitrocellulose filter membranes as previously described [24]. Anti-GLUT-4 polyclonal antibody R 820 and anti-GLUT-1 polyclonal antibody α G6 were used at 1:500 and 1:100 dilutions, respectively. Autoradiographs were quantitated by laser scanning densitometry.

2.5. *Cytochalasin B binding*

D-Glucose-protectable binding of cytochalasin B is recognized as a confident measurement for the estimation of glucose transporters [25]. The binding of [^{3}H]cytochalasin B was measured at equilibrium with 0.2 μM cytochalasin B (saturating concentration) in the presence of 5.5 μM cytochalasin E and 200 mM of either D- or L-glucose as previously described [26,27]. [^{3}H]Cytochalasin B binding was assayed in triplicate. Membranes were separated from the medium by rapid (2 s) filtration through GF/B Whatman filters (Whatman, Clifton, NJ) as reported earlier [26,27]. Results were analyzed statistically by Student's t-test for unpaired data.

3. RESULTS

The effects of exercise, insulin and combined exercise⟶insulin treatments on glucose uptake into perfused rat hindlimbs is shown in fig.1. Insulin perfusion (1000 μU/ml for 20 min) or acute exercise (45 min treadmill run), induced 5-fold and 3.2-fold increases in glucose uptake, respectively. However, when the bout of exercise was followed by insulin perfusion, a 7-fold increase in glucose uptake was observed.

Plasma membranes and intracellular membranes prepared from control, exercise- and exercise⟶insulin-treated rat hindlimb muscles, were subjected to SDS-PAGE and Western blot analysis with anti GLUT-4 antibody (fig.2). This antibody reacted

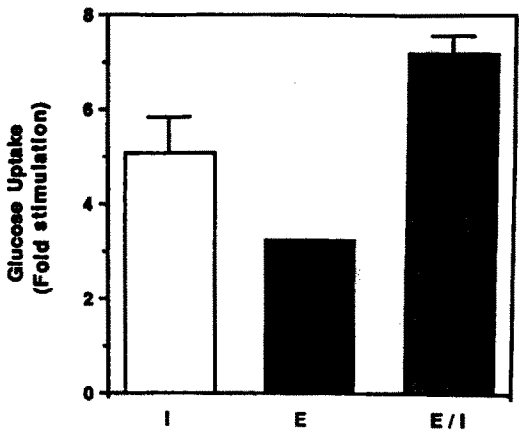

Fig.1. Glucose uptake (fold stimulation) in perfused rat hindlimb. I = insulin- (1000 μU/ml for 20 min) stimulated uptake; E = exercise- (45 min treadmill run) stimulated uptake; E/I = exercise⟶insulin- (45 min treadmill run followed by 1000 μU/ml insulin for 20 min) stimulated uptake. For exercise (E), the SE was smaller than the symbol.

positively with a band of 46 kDa in both plasma membranes and intracellular membranes. Both of these membrane fractions contained similar amounts of GLUT-4 transporters/mg protein. Intracellular membranes contained 7-fold more GLUT-4 transporters than plasma membranes when the protein yields of each fraction were considered. Quantification of immunoblots by laser scanning densitometry showed that both exercise and exercise⟶insulin treatments caused significant and comparable increases, 2.5-fold and 2.75-fold, respectively (means of 3 independent experiments), in GLUT-4 transporters in the plasma membrane, relative to the unstimulated control. Moreover, exercise did not cause any significant decrease in GLUT-4 in the intracellular membrane in 5

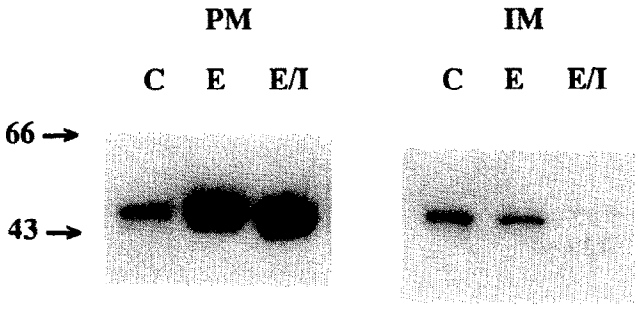

Fig.2. Effect of exercise and exercise⟶insulin treatment on the subcellular distribution of the GLUT-4 glucose transporter in skeletal muscle membranes. Plasma membranes (PM) and intracellular membranes (IM) were prepared from control (C), exercised (E) and exercise⟶insulin (E/I) treated rat hindlimb muscles. Membranes (30 μg) were separated on 12% polyacrylamide gels, transferred to nitrocellulose paper and immunoassayed by Western blot analysis using anti-GLUT-4 antibody, as described under section 2. The numbers on the left indicate the position of the molecular mass markers, in kDa. One representative experiment performed 3–5 times with independent membrane preparations is shown.

independent experiments, while exercise⟶insulin treatment caused a 53% decrease in GLUT-4 in this membrane fraction. Insulin alone increased GLUT-4 transporters in the plasma membrane by 2.6-fold and caused a 30% reduction in GLUT-4 in the intracellular membrane fraction [29].

Since addition of insulin after exercise further increased glucose uptake but did not significantly increase plasma membrane GLUT-4 transporters (relative to exercise-recruited GLUT-4 transporters), we considered the possibility that insulin might increase plasma membrane GLUT-1 transporters. Anti-GLUT-1 antibody reacted positively with a band of 46 kDa in plasma membranes and intracellular membranes (fig.3). In contrast to the subcellular distribution of GLUT-4, a higher density of GLUT-1 transporters was found per mg protein in the plasma membrane than the intracellular membrane. When the protein yield of each fraction was considered, the plasma membrane was found to contain 2.2-fold more GLUT-1 transporters than intracellular membranes, showing that in absolute amounts, GLUT-1 transporters are also more abundant in the plasma membrane. More importantly, quantification by laser scanning densitometry showed that neither exercise nor exercise⟶insulin treatments caused any significant changes in the subcellular distribution of this transporter isoform (fig.3).

The observation that both exercise and exercise⟶insulin treatments cause a comparable increase in GLUT-4 transporters in the plasma membrane is in contrast to the further increase in glucose uptake caused by exercise⟶insulin treatment relative to exercise alone (fig.1). To confirm this finding, we determined the total number of glucose transporters (irrespective of isoform) in the plasma membrane of control, exercise- and exercise⟶insulin-treated rats using D-glucose-protectable cytochalasin B binding (fig.4). Exercise and exercise⟶insulin caused similar increases in glucose transporter number, 1.65- and 1.77-fold, respectively. This observation parallels the results of immunoblotting with the anti-GLUT-4 antibody (fig.3). The lesser increase of cytochalasin B binding sites relative to that of GLUT-4 transporters, is probably due to the reaction of cytochalasin B with GLUT-1 transporters in addition to GLUT-4 transporters in the plasma membrane.

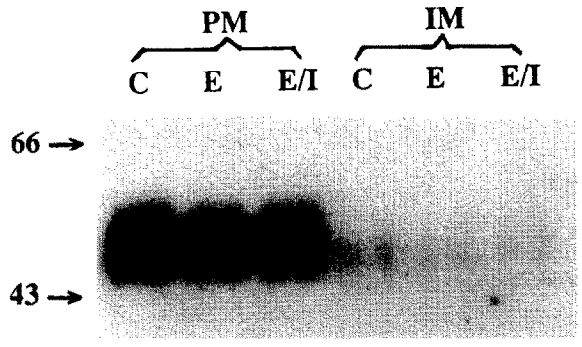

Fig.3. Effect of exercise and exercise⟶insulin treatment on the subcellular distribution of the GLUT-1 glucose transporter in skeletal muscle membranes. Plasma membranes (PM) and intracellular membranes (IM) were prepared from control (C), exercised (E) and exercise⟶insulin (E/I) treated rat hindlimb muscles. Membranes (30 μg) were separated on 12% polyacrylamide gels, transferred to nitrocellulose paper and immunoassayed by Western blot analysis using anti-GLUT-1 antibody, as described under section 2. The numbers on the left indicate the position of the molecular mass markers, in kDa. One representative experiment performed 3–5 times with independent membrane preparations is shown.

4. DISCUSSION

Muscular contraction stimulates glucose uptake in insulin-depleted rat epitrochlearis muscles [3,6] and rat hindlimbs [4], suggesting that the mechanism of exercise-induced enhancement of glucose uptake is independent of insulin. Moreover, the maximal stimulations by insulin and contractile activity are additive [3,6]. These observations have led to the proposal that insulin and contractile activity stimulate glucose transport via two independent mechanisms [3,6]. The glucose uptake measurements in the present study (fig.1) show that following exercise, insulin treatment further increased glucose uptake in rat hindlimb skeletal muscles, in agreement with the earlier reports [3,6]. Here we show that both exercise and exercise⟶insulin treatments increased plasma membrane GLUT-4 transporters but had no effect on the subcellular distribution of GLUT-1. Exercise alone did not decrease GLUT-4 transporters in the intracellular membrane fraction. In contrast, addition of insulin after exercise resulted in a marked decrease in GLUT-4 transporters in this fraction. Similar results have been obtained with insulin alone [29].

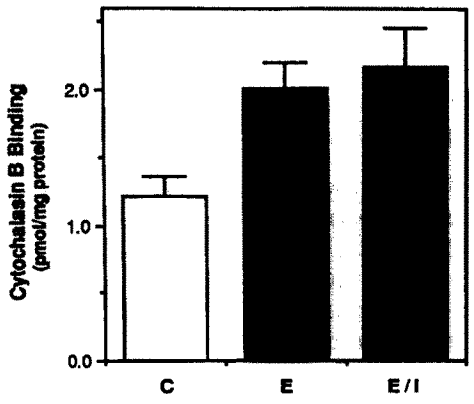

Fig.4. Cytochalasin B binding to plasma membranes from control (C), exercise- (E), and exercise⟶insulin-treated (E/I) rat hindlimb muscles. D-Glucose-protectable cytochalasin B binding to membranes was measured as described under section 2. Bars are the mean ± SE of 6 independent preparations each assayed in triplicate. $P < 0.01$ for E vs C and for E/I vs C.

Stimulation of glucose uptake in rat hindlimb muscles by contractile activity persists for a considerable period of time after contraction has stopped [2,3]. Under exercise⟶insulin conditions, the muscles are first exercised before perfusion with insulin. Hence the initial increase in plasma membrane glucose transporters would arise from the 'exercise-recruitable' transporter pool. We had anticipated that subsequent insulin treatment would further increase GLUT-4 transporters in the plasma membrane due to transporter recruitment from the 'insulin-sensitive' intracellular transporter pool. However, while insulin reduced the number of GLUT-4 transporters in the intracellular membrane of exercised animals, there was no further increase in plasma membrane GLUT-4 transporters caused by exercise⟶insulin treatment compared to exercise alone. We speculate that there may be a maximum amount of GLUT-4 transporters recruited to the plasma membrane (about a 2.5-fold increase), and that following exercise, insulin causes migration of GLUT-4 transporters to a fraction other than the plasma membrane.

In addition, it is intriguing that while there is a clear 'additive effect' on glucose uptake by the exercise⟶insulin treatment relative to each independent stimulus, there is no additive effect in the total number of glucose transporters in the plasma membrane (measured by cytochalasin B binding) or in the density of GLUT-4 transporters (assessed by immune staining). One possible explanation is that, following exercise, insulin may increase the intrinsic activity of plasma membrane glucose transporters (GLUT-4 and/or GLUT-1). Recent reports have suggested that insulin treatment alone increases the intrinsic activity of glucose transporters in rat skeletal muscles [16,28]. Moreover, Sternlicht et al. [28] suggested that the transporters present in the plasma membrane after exercise⟶insulin treatment have a higher intrinsic activity than after insulin treatment alone. This was based on the observation that glucose transport activity was identical in plasma membrane vesicles prepared from exercise-, insulin-, and exercise⟶insulin-treated rat skeletal muscles, although plasma membranes of exercise⟶insulin-treated rat muscles showed a 40% lower increase in cytochalasin B binding sites compared to plasma membranes of exercised rat muscles.

In conclusion, the results of the present study show that following exercise, insulin addition decreases GLUT-4 transporters in an intracellular membrane fraction, without increasing them significantly in the plasma membrane, relative to exercise alone. This suggests that exercise might modulate insulin-induced transporter recruitment in skeletal muscles. In addition, the enhancement of glucose uptake caused by this exercise⟶insulin treatment, relative to exercise or insulin treatment alone, is not coincident with a further increase in plasma membrane transporter number. This suggests that the increase in transporter intrinsic activity caused by insulin is magnified after exercise.

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