Clenbuterol prevents epinephrine from antagonizing insulin-stimulated muscle glucose uptake

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Hunt, Desmond G., Zhenping Ding, and John L. Ivy. Clenbuterol prevents epinephrine from antagonizing insulin-stimulated muscle glucose uptake. J Appl Physiol 92: 1285–1292, 2002. First published November 30, 2001; 10.1152/japplphysiol.01009.2001.—In the present study, we investigated the effects of chronic clenbuterol treatment on insulin-stimulated glucose uptake in the presence of epinephrine in isolated rat skeletal muscle. Insulin (50 μU/ml) increased glucose uptake in both fast-twitch (epitrochlearis) and slow-twitch (soleus) muscles. In the presence of 24 nM epinephrine, insulin-stimulated glucose uptake was completely suppressed. This suppression of glucose uptake by epinephrine was accompanied by an increase in the intracellular concentration of glucose 6-phosphate and a decrease in insulin-receptor substrate-1-associated phosphatidylinositol 3-kinase (IRS-1/PI3-kinase) activity. Clenbuterol treatment had no direct effect on insulin-stimulated glucose uptake. However, after clenbuterol treatment, epinephrine was ineffective in attenuating insulin-stimulated muscle glucose uptake. This ineffectiveness of epinephrine to suppress insulin-stimulated glucose uptake occurred in conjunction with its inability to increase the intracellular concentration of glucose 6-phosphate and attenuate IRS-1/PI3-kinase activity. Results of this study indicate that the effectiveness of epinephrine to inhibit insulin-stimulated glucose uptake is severely diminished in muscle from rats pretreated with clenbuterol.

In an effort to discern the effects of chronic β-adrenergic-receptor stimulation on glucose uptake and disposal, our laboratory chronically administered the β-adrenergic agonist clenbuterol to rats for 5 wk (39, 40). During in vivo experiments, chronic clenbuterol treatment led to a reduction in plasma insulin levels and improved glucose tolerance (39). Similarly, our laboratory observed an increase in insulin-stimulated glucose clearance and skeletal muscle glucose uptake by using the euglycemic-hyperinsulinemic clamp (29). In contrast, our laboratory observed no increase in insulin-stimulated glucose uptake or disposal in skeletal muscle during in situ experiments by using the hindlimb perfusion technique or in vitro by using an isolated muscle preparation (39–41). This raised the question as to the mechanism by which chronic clenbuterol administration improves insulin-stimulated glucose uptake in skeletal muscle in vivo but not in vitro.

Chronic activation of β-adrenergic receptors leads to desensitization and subsequent reduction of β-adrenergic-receptor number at the plasma membrane, which renders skeletal muscle resistant to the action of epinephrine (12). Knowing that the actions of epinephrine are antagonistic to those of insulin, we considered the possibility that the increase in skeletal muscle insulin sensitivity observed in vivo after chronic clenbuterol administration might be due to a reduced influence of epinephrine over insulin action as a result of β-adrenergic-receptor downregulation. In the present study, we address this possibility indirectly by testing the hypothesis that chronic exposure to clenbuterol will attenuate the effect of epinephrine on insulin-stimulated muscle glucose uptake in vitro. Results of this study indicate that the effectiveness of epinephrine to inhibit insulin-stimulated glucose uptake is severely diminished in muscle from rats pretreated with clenbuterol or when β-adrenergic receptors are blocked. Furthermore, this attenuation in the action of epinephrine was accompanied by an enhanced activation of the insulin signaling protein phosphatidylinositol 3-kinase (PI3-kinase).

POSTPRANDIAL GLUCOSE HOMEOSTASIS is largely dependent on the ability of skeletal muscle to take up and dispose of blood glucose. Two notable hormones that regulate skeletal muscle glucose uptake are the pancreatic hormone insulin and the adrenal hormone epinephrine. Insulin, acting via the insulin receptor, increases glucose uptake by activating glucose transport and glycogen storage (1, 6, 14), whereas epinephrine, acting via β-adrenergic receptors, attenuates these processes (1, 4, 16, 19, 31, 33). However, chronic exposure to β-adrenergic agonists has been found to improve insulin action and glucose clearance in rats and humans (25, 35). From these studies, it was suggested that skeletal muscle was responsible for the improvement in glucose clearance, although the direct contribution of skeletal muscle was not assessed (25, 35).

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METHODS

Animals and muscle preparation. Female Sprague-Dawley rats (n = 60), weighing between 110 and 120 g, were randomly assigned to the following four groups: basal, insulin, insulin-epinephrine, and insulin-epinephrine-propranolol. In addition, 30 Sprague Dawley rats, weighing between 35 and 40 g, were administered clenbuterol and randomly assigned to either an insulin or insulin-epinephrine group. Rats treated with clenbuterol (dissolved in deionized water) were gavaged with 0.5 mg clenbuterol/kg body weight, once a day, 5 days/wk, for 3 wk. The length of clenbuterol administration provided sufficient time for downregulation of the β-adrenergic receptors and for the clenbuterol-treated rats to reach a body mass of 110–120 g. Only the clenbuterol-treated rats were gavaged, because our laboratory has previously shown that the effects of clenbuterol are not dependent on the manner in which it is administered (29, 39, 41). All rats were obtained from and housed in the Animal Resource Center, University of Texas at Austin (Austin, TX). They were allowed free access to standard rat chow and water up to 8 h before euthanasia. The temperature of the animal room was maintained at 21°C, and a 12:12-h light-dark cycle was set. All procedures were approved by the Animal Care and Use Committee of the University of Texas and conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, Bethesda, MD 20892].

All rats were anesthetized after an 8-h fast via an intraperitoneal injection of pentobarbital sodium (6.5 mg/100 g body wt). Rats pretreated with clenbuterol received anesthesia 48 h after their last clenbuterol treatment. Once the rats were anesthetized, the epitrochlearis (fast-twitch) and soleus (slow-twitch) muscles were excised. The soleus muscle was separated into strips weighing ~15 mg, with the epitrochlearis used to assess glucose uptake, glycogen, glucose 6-phosphate, and PI3-kinase activity after in vitro incubation under the treatment conditions previously outlined. The epitrochlearis muscle was chosen for this study because of its thin diameter and predominately fast-twitch fiber composition: 15% type I, 20% type IIA, and 65% type IIB (15). The soleus muscle was chosen for the study because of the ability to longitudinally section this tubular muscle into 15- to 20-mg strips, which can be incubated with minimal limitation due to diffusion and its predominantly slow-twitch fiber composition: 84% type I, 16% type IIA, and 0% type IIB (15).

Muscle incubation. After isolation, epitrochlearis and soleus muscles were individually preincubated for 50 min at 29°C in 1.5 ml of continuously gassed (95% O2-5% CO2) Krebs-Henseleit bicarbonate buffer containing 0.1% BSA, 32 mM mannitol, and 8 mM glucose. After the preincubation, muscles were washed for 10 min in fresh buffer (1.5 ml) containing 0.1% BSA and 40 mM mannitol. Muscles were then transferred to fresh buffer, and glucose uptake was measured in the presence of 2 mM pyruvate, 6 mM glucose, 280 μCi/mmol 2-[3H]deoxyglucose (2-DG; Du Pont NEN, Boston, MA), 32 mM mannitol, 10 μCi/mmol [14C]mannitol (ICN Pharmaceuticals, Costa Mesa, CA), and 0.5 mg/ml ascorbic acid with either 0 insulin, 50 μU/ml insulin (Eli Lilly, Indianapolis, IN), 50 μU/ml insulin plus 24 nM epinephrine (Sigma Chemical, St. Louis, MO), or 50 μU/ml insulin plus 24 nM epinephrine plus 10 μM propranolol (Sigma Chemical). Muscles were then incubated at 29°C in the uptake medium for 30 min. After the last incubation period, muscles were blotted and freeze clamped with Wollenberg tongs cooled in liquid nitrogen. Glycogen, glucose 6-phosphate, and PI3-kinase activity were measured in muscle incubated in the absence of radioactive isotopes.

The concentration of epinephrine was selected because it represents a physiological concentration normally observed in the rat during light-to-moderate stress (32). The insulin concentration used was selected because it represents a normal physiological concentration. In addition, preliminary investigation established that 50 μU/ml insulin elicited a glucose uptake rate significantly above basal and that a clear effect of epinephrine on glucose uptake could be detected. Furthermore, insulin receptor substrate-1 (IRS-1)-associated PI3-kinase (IRS-1/PI3-kinase) activities in the epitrochlearis and soleus muscles were found to be maximal after 30 min of incubation with 50 μU/ml insulin (Fig. 1).

Muscle processing for determination of glucose uptake. Glucose uptake was estimated by determining the incorporation rate of 2-DG into skeletal muscle (26, 34). 2-DG is a glucose analog that has uptake rates similar to glucose and thus provides a good estimate of the rate of glucose uptake in skeletal muscle. Incubated muscles were weighed and dissolved in 1 M KOH for 15 min at 60°C. Dissolved samples were then neutralized with 1 M HCl, and 0.5 ml of each supernatant was added to 6 ml of Biosafe II scintillation fluid (Research Products International, Mount Prospect, IL). Samples were counted for 3H and 14C in an LS-6000 liquid scintillation spectrophotometer (Beckman, Fullerton, CA).

Muscle glycogen determination. Muscle glycogen concentration was determined after complete enzymatic degradation to glucose with amyloglucosidase (30). An aliquot of the KOH-digested muscle was incubated overnight in 0.3 M sodium acetate buffer, pH 4.8, which contained 5 mg/ml amyloglucosidase (Boehringer Mannheim, Mannheim, Germany). Liberated glucose was then measured by using a spectrophotometric Trinder reaction (Sigma Chemical).

Glucose 6-phosphate determination. Muscle samples were added to 300 μl of 10% perchloric acid and homogenized at 0°C. The supernatant was neutralized with saturated (30%) KHCO3. Neutralized samples were then centrifuged for 15 min and assayed for glucose 6-phosphate according to Lowry and Passonneau (24).

Fig. 1. Insulin-stimulated (50 μU/ml) insulin-receptor substrate-1-associated phosphatidylinositol 3-kinase (IRS-1/PI3-kinase) activity time course for the epitrochlearis and soleus muscle. Values are means ± SE in given in counts/min (CPM). *Significantly different from basal, P < 0.05.
Table 1. Glycogen, ATP, and creatine phosphate in the epitrochlearis and soleus muscle

<table>
<thead>
<tr>
<th></th>
<th>Epitrochlearis</th>
<th>Soleus</th>
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<tbody>
<tr>
<td>Glycogen, μmol/g</td>
<td>20.79 ± 1.05</td>
<td>21.56 ± 0.50</td>
</tr>
<tr>
<td>Preincubation</td>
<td>22.76 ± 0.95</td>
<td>24.67 ± 1.21</td>
</tr>
<tr>
<td>ATP, μmol/g</td>
<td>5.07 ± 0.54</td>
<td>4.73 ± 0.82</td>
</tr>
<tr>
<td>Preincubation</td>
<td>5.47 ± 0.70</td>
<td>5.19 ± 0.83</td>
</tr>
<tr>
<td>Creatine phosphate, μmol/g</td>
<td>26.24 ± 0.88</td>
<td>21.68 ± 2.27</td>
</tr>
<tr>
<td>Preincubation</td>
<td>29.64 ± 1.7</td>
<td>22.26 ± 1.80</td>
</tr>
</tbody>
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Values are means ± SE; n = 5 samples per muscle type. Concentrations are determined from isolated muscles before and after the incubation procedure.

RESULTS

Validation of isolated muscle preparation. To test the viability of the isolated muscle preparation, glycogen, ATP, and creatine phosphate were measured pre- and postincubation. As indicated in Table 1, no differences in pre- and postincubation glycogen, ATP, or creatine phosphate were found for the epitrochlearis or soleus muscle. These results suggest a viable isolated muscle preparation.

Effect on glucose uptake. A physiological concentration (50 μU/ml) of insulin increased glucose uptake by 100% above basal in the epitrochlearis and by 97% above basal in the soleus muscle (Figs. 2 and 3). Clenbuterol treatment did not augment insulin-stimulated glucose uptake in the epitrochlearis or soleus muscles. Epinephrine (24 nM) completely inhibited insulin-stimulated glucose uptake in both the epitrochlearis and soleus muscles. This reduction in insulin-stimulated glucose uptake by epinephrine was attenuated by the β-adrenergic antagonist propranolol and in skeletal muscle pretreated with clenbuterol.

Effect on glycogen concentration. Insulin increased glycogen concentration above basal in the epitrochlearis (56%) (Fig. 4). Clenbuterol treatment did not augment insulin-stimulated glycogen concentration in the epitrochlearis. Epinephrine stimulation significantly reduced muscle glycogen concentration in the epitrochlearis muscle compared with muscle exposed to only insulin. This reduction in glycogen concentration by epinephrine was prevented by the β-adrenergic antagonist propranolol and by clenbuterol treatment. Nei-

![Fig. 2. Glucose uptake in the epitrochlearis muscle. Values are means ± SE. Ins, insulin; Ins/Epi, insulin and epinephrine; Ins/Epi/Pro, insulin, epinephrine, and propranolol; Ins/Clen, insulin and clenbuterol; Ins/Clen/Epi, insulin, clenbuterol, and epinephrine. *Significantly different from basal, P < 0.05. †Significantly different from insulin, P < 0.05. §Significantly different from insulin and epinephrine, P < 0.05.](http://jap.physiology.org/01/10.2203/32.2)
other insulin nor epinephrine had an effect on muscle glycogen concentration of the soleus (Fig. 5). However, in solei from clenbuterol-pretreated rats, muscle glycogen was significantly greater than in muscle exposed to insulin only.

**Effect on glucose 6-phosphate concentration.** Insulin stimulation increased glucose 6-phosphate by 80% in the epitrochlearis and by 250% in the soleus (Table 2). Simultaneous stimulation with insulin and epinephrine increased glucose 6-phosphate in the epitrochlearis and soleus to values 150 and 540% above basal, respectively. The increase in glucose 6-phosphate by epinephrine was completely blocked with the β-adrenergic-antagonist propranolol and with chronic clenbuterol treatment. The concentrations of glucose 6-phosphate in the propranolol- and clenbuterol-pretreated solei were significantly less than with insulin stimulation alone.

**Effect on IRS-1/PI3-kinase activity.** Insulin stimulation significantly increased IRS-1/PI3-kinase activity in the epitrochlearis by 120% and in the soleus by 320% (Figs. 6 and 7). However, epinephrine completely inhibited the activation of IRS-1/PI3-kinase activity by insulin. This inhibition in PI3-kinase activity by epinephrine was completely blocked by propranolol and...
by pretreatment with clenbuterol in the epitrochlearis but was only partially blocked in the soleus.

**DISCUSSION**

A rise in plasma insulin concentration increases the rate of glucose uptake in skeletal muscle. This increase in insulin-stimulated glucose uptake is promoted by an increase in glucose transport, glucose oxidation, and glycogen synthesis (27, 28). Acute exposure to the insulin antagonist epinephrine, acting via β2-adrenergic receptors, decreases the ability of insulin to stimulate glucose uptake (6, 16, 21). However, chronic exposure to certain β2-adrenergic agonists has been shown to increase insulin-stimulated glucose clearance in vivo (25, 35). In a recent study from our laboratory, an increase in insulin-stimulated glucose clearance was observed in vivo after chronic treatment with the β2-adrenergic agonist clenbuterol (29). Tissue analysis revealed that the major tissue responsible for the clearance of blood glucose was skeletal muscle (29).

In the present study, we were unable to demonstrate an increase in insulin-stimulated glucose uptake in isolated skeletal muscle from rats chronically treated with clenbuterol for 3 wk. This result is in agreement with previous in vitro studies from our laboratory, in which chronic clenbuterol treatment had no effect on insulin-stimulated glucose uptake during hindlimb perfusions (39, 41). These results suggest that chronic clenbuterol administration does not amplify the ability of insulin to stimulate glucose uptake in skeletal muscle. However, this does not explain the discrepancy in insulin-stimulated glucose uptake observed in vivo and in vitro with chronic clenbuterol treatment.

Chronic exposure to clenbuterol leads to downregulation of β2-adrenergic receptors in skeletal muscle (12, 40), which causes skeletal muscle to become resistant to the action of epinephrine (9, 12). Because of the antagonistic effects of epinephrine on insulin action, we decided to examine its in vitro effects on insulin-stimulated glucose uptake and metabolism in skeletal muscle from clenbuterol-treated rats. We reasoned that the downregulation of β2-adrenergic receptors could possibly reduce the effectiveness of epinephrine while increasing the action of insulin in vivo.

In the presence of a physiological insulin concentration, 24 nM epinephrine were found to completely inhibit insulin-stimulated glucose uptake in isolated skeletal muscle. This demonstrates that a physiological epinephrine concentration will suppress insulin-stimulated glucose uptake and implies that small changes in plasma epinephrine concentration may have major effects on insulin action in vivo. However, epinephrine had no attenuating effect on insulin-stimulated glucose uptake in skeletal muscle from rats chronically treated with clenbuterol. This result suggests that the ability of clenbuterol to augment insulin-stimulated glucose uptake in vivo may be due to the inability of endogenous epinephrine to antagonize insulin action. This is supported by our finding that the antagonistic effect of epinephrine on insulin action was also blocked by exposure of incubated muscle to the β2-adrenergic-receptor antagonist propranolol.

Several mechanisms have been proposed to explain the antagonistic effects of epinephrine on insulin-stimulated glucose uptake. Epinephrine is a known activa-
tor of glycogenolysis in skeletal muscle (6, 16). The increase in glycogenolysis induced by epinephrine leads to the accumulation of the intracellular metabolite glucose 6-phosphate, which inhibits hexokinase activity and glucose phosphorylation (14). Under conditions of rapid glucose transport, such as during insulin stimulation, inhibition of hexokinase by epinephrine would result in an increase in intracellular free glucose, causing an increase in the countertransport of glucose from the cell and reducing the rate of glucose clearance from the extracellular medium.

In the present study, epinephrine had contrasting effects on glycogenolysis in different muscle fiber types during insulin stimulation. In the fast-twitch epitrochlearis, we observed a 25% reduction in glycogen, whereas no significant reduction was observed in the slow-twitch soleus. These results corroborate the findings of previous studies, where epinephrine exposure had a significant effect on glycogenolysis in fast-twitch fibers but not slow-twitch fibers (17). This difference in glycogenolysis between fiber types during epinephrine stimulation has been attributed to differences in glycogen concentration and phosphorylase activity (17). Despite no detectable glycogenolysis in the soleus, during insulin stimulation, epinephrine significantly increased the intracellular concentration of glucose 6-phosphate in both muscle fiber types. Glucose 6-phosphate was elevated to levels known to inhibit hexokinase activity in skeletal muscle. These findings support earlier studies that suggested a glucose-6-phosphate-dependent mechanism by which epinephrine inhibits insulin-stimulated glucose uptake in skeletal muscle via the inhibition of hexokinase (6, 14). Furthermore, in muscle from rats chronically treated with clenbuterol or exposed to propranolol, the epinephrine-induced increase in glucose 6-phosphate was blocked. Thus the inability of epinephrine to increase glucose 6-phosphate, possibly due to inhibition of glycogenolysis, may explain the mechanism by which clenbuterol treatment prevents the attenuation of insulin-stimulated glucose uptake in skeletal muscle.

It is also possible that epinephrine reduces insulin-stimulated glucose uptake by reducing the activation of key insulin-signaling proteins involved in glucose transport. One protein that has been linked to the activation of glucose transport is PI3-kinase. Several lines of evidence have indicated that the activation of PI3-kinase is required for the activation of insulin-stimulated glucose transport (5, 7, 10).

In the present study, insulin stimulation increased IRS-1/PI3-kinase activity in skeletal muscle. This result is in agreement with earlier studies, in which insulin was shown to increase IRS-1/PI3-kinase activity (36, 38). In contrast, epinephrine completely blocked the ability of insulin to stimulate IRS-1/PI3-kinase activity in skeletal muscle. This is the first study to demonstrate that epinephrine can attenuate insulin-stimulated IRS-1/PI3-kinase activity. The mechanism by which epinephrine reduces insulin-stimulated IRS-1/PI3-kinase activity is unknown. However, agents that increase intracellular cAMP also increase the phosphorylation of the insulin receptor on serine/threonine residues, which has been shown to reduce the receptor's tyrosine kinase activity (37). Thus inhibition of insulin-stimulated muscle glucose uptake by epinephrine may be due to inhibition glucose transport as a consequence of serine/threonine phosphorylation of the insulin receptor and attenuation of IRS-1/PI3-kinase activation. The effect of epinephrine on insulin-stimulated muscle glucose transport, however, is equivocal at this time and requires further investigation (6, 13, 20).

In skeletal muscle from rats pretreated with clenbuterol, epinephrine had no effect on insulin-stimulated IRS-1/PI3-kinase activity in the fast-twitch epitrochlearis. In the slow-twitch soleus, epinephrine was able to produce a small reduction in insulin-stimulated IRS-1/PI3-kinase activity. However, this small reduction in IRS-1/PI3-kinase activity had no inhibiting effect on insulin-stimulated glucose uptake in the soleus muscle of clenbuterol-treated rats. These results raise the possibility that chronic clenbuterol treatment increases insulin-stimulated glucose uptake in vivo by reducing the effectiveness of epinephrine to inhibit intracellular insulin signaling. These results also suggest that full activation of IRS-1/PI3-kinase is not necessary to attain normal activation of insulin-stimulated glucose uptake.

To our knowledge, this is the first study to examine IRS-1/PI3-kinase activity in vitro with a physiological insulin (50 μU/ml) concentration. Insulin increased IRS-1/PI3-kinase activity in both the epitrochlearis and soleus. Whereas IRS-1/PI3-kinase was increased in both muscle fiber types, both the rate and magnitude of increase differed. The magnitude of increase in PI3-kinase activity of the soleus was threefold greater than the activity of the epitrochlearis. This difference in insulin-stimulated PI3-kinase activity between muscle fiber types is in agreement with the recent observations by Song et al. (36), using a maximally-stimulating concentration of insulin. However, Song et al. reported no difference in time to peak activity in the epitrochlearis and soleus muscles, whereas we found that peak activity was reached much sooner in the epitrochlearis. These results suggest that this difference in temporal response of insulin-stimulated PI3-kinase activity to insulin between fast- and slow-twitch muscle fibers is insulin concentration dependent.

It is well established that there are fiber-type differences in insulin-stimulated glucose uptake (3, 8). Skeletal muscle composed of predominately slow-twitch fibers displays greater responsiveness to insulin compared with skeletal muscle composed of predominately fast-twitch fibers (3, 9). This difference in responsiveness in glucose uptake between fiber types has been attributed to differences in GLUT-4 protein concentration (2, 11). However, recent results, as well as those of the present study, suggest that there are also differences in the concentration and activity of the various insulin-signaling proteins between fiber types, with slow-twitch fibers having the greater protein concentration and total activity (36). Therefore, the differ-
ences in insulin-stimulated glucose uptake observed between the slow-twitch soleus and fast-twitch epitrochlearis may be due, in part, to differences in concentration and activity of the various insulin-signaling proteins.

In the present study, we have tried to determine the cause for discrepancy in insulin-stimulated muscle glucose uptake observed in vivo and in vitro after chronic clenbuterol administration. When studied in vivo, there is a clear and obvious increase in insulin-stimulated glucose uptake in skeletal muscle from rats pretreated with clenbuterol (29). In the present study, clenbuterol treatment had no effect on insulin-stimulated glucose uptake in vitro. However, clenbuterol treatment did prevent epinephrine from attenuating insulin-stimulated glucose uptake in vitro, suggesting that the effectiveness of epinephrine to inhibit insulin-stimulated glucose uptake is severely diminished in muscle from rats pretreated with clenbuterol. Thus a possible explanation for the increase in insulin-stimulated glucose uptake in skeletal muscle in vivo after chronic clenbuterol treatment may be the ineffectiveness of epinephrine to antagonize the action of insulin.

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REFERENCES


