Metformin treatment enhances insulin-stimulated glucose transport in skeletal muscle of Sprague-Dawley rats

Stephen E. Borst, Ph.D.*, Harold G. Snellen, M.S., Hsiao Ling Lai, B.S.

Departments of Pharmacology and Exercise and Sport Sciences, University of Florida and Geriatric Research, Education and Clinical Center, VA Medical Center, Gainesville, FL 32608-1197, USA

Abstract

Although the glucose-lowering properties of metformin are well-established, its effects on glucose metabolism in skeletal muscle have not been clearly defined. We tested the effects of metformin in young adult male Sprague-Dawley rats, which have a documented reduced response to insulin in skeletal muscle. Rats were treated with metformin for 20 days (320 mg/kg/day) in the drinking water. During this period, metformin completely prevented the increase in food intake and decreased adiposity by 30%. Metformin also reduced insulin secretion by 37% following an intra-peritoneal injection of glucose. Finally, metformin enhanced transport of [^{3}H]-2-deoxyglucose in isolated strips of soleus muscle. Metformin substantially increased insulin-stimulated transport, while having no effect on basal transport. In control rats, a maximal concentration of insulin stimulated transport 77% above basal. In metformin-treated rats, insulin stimulated transport 206% above basal. We conclude that in the Sprague-Dawley rat model, metformin causes a significant increase in insulin-responsiveness. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Metformin; Glucose transport; Skeletal muscle; Sprague-Dawley rat; Insulin

Introduction

Metformin is a euglycemic agent which reduces hyperglycemia through a number of known mechanisms (1–3). Although metformin inhibits both intestinal absorption and hepatic production of glucose, these actions are not sufficient to explain its anti-hyperglycemic effect (2). Probably the most important actions of metformin stem from its ability to enhance insulin-stimulated glucose uptake in a variety of tissues, including adipose tissue, skeletal muscle and smooth muscle (1, 2, 4). The precise mechanism of metformin action in skeletal muscle is not known and may involve either glucose transport or glucose utilization subse-

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* Corresponding author. Stephen Borst, Ph.D., V.A. Medical Center, GRECC–182, 1601 SW Archer Rd., Gainesville, FL 32608-1197, USA. Tel.: 352-374-6114; fax: 352-374-6142.
   E-mail address: sborst@pharmacology.ufl.edu (S.E. Borst)

quent to transport. *In vitro*, metformin enhances glucose transport in strips of skeletal muscle derived from insulin-resistant human subjects, but this enhancement occurs only at metformin concentrations that are 10-fold higher than are clinically observed (5). Animal studies of chronic metformin administration have failed thus far to detect more than a modest increase in muscle glucose transport (1, 6, 7) However most of these studies were not performed with animals with impaired insulin responses. For the present experiments, we chose to study young adult male Sprague-Dawley rats. Using a perfused hindlimb preparation, Goodman *et al*. (8) have shown that these animals develop an impaired response to insulin in skeletal muscle during maturation, with significant reductions in both the sensitivity and efficacy of insulin. Our objectives were to determine the effects of metformin administration on food intake, adiposity, whole-body insulin-sensitivity and insulin-stimulated glucose transport in soleus muscle.

Materials and Methods

Study design

Metformin (Sigma, St. Louis MO) was administered at a dose of 320 mg/kg/day to male Sprague-Dawley rats in the drinking water for 20 days. A similar dosing regimen has been shown to reduce food intake and hyperinsulinemia in Zucker rats (9–11). Our rats were aged 14 weeks at the end of the experiment, an age at which the response to insulin in skeletal muscle is already substantially impaired. (8). Water intake was measured in experimental and control animals before and twice during the experiment. The concentration of metformin was adjusted as needed to maintain the dose. Metformin had no affect on water consumption. Food intake was measured before metformin administration and after 10 and 20 days. Food baskets were weighed to determine 24 hr. food intake on each of 2 successive days. Measurements were made at 10 AM, when food intake was low. Small food particles produced by chewing may end up in the bedding. This was measured in a separate, prior experiment and found to less than 0.1 gm/day. Glucose tolerance was measured after 20 days of metformin treatment. One day later, rats were sacrificed for measurement of glucose transport in isolated strips of skeletal muscle.

Glucose tolerance

Glucose tolerance was measured by the method of Chen *et al*. (12), briefly as follows. Rats were fasted 24 hr and received a glucose load of 3.0 g/kg, i.p. Blood was sampled by tail nick before glucose loading and at 30, 60 and 120 minutes after. Plasma glucose levels were determined by the Trinder method (Sigma Chemical Co., St. Louis MO) and insulin was measured by radioimmunoassay, using rat insulin as a standard (Linco Research, Inc. St. Louis MO).

Muscle glycogen determination

Muscles were dissolved In 30% KOH and glycogen extracted by the method of Reddi and Jyothirmayi (13). Glycogen was assayed by absorbance at 620 nm in 0.2% anthrone and concentrated sulphuric acid by the method of Yang (14). Glycogen content was measured in

three separate 100 mg samples of gastrocnemius muscle. Results were averaged and expressed as mg glycogen/g muscle wet weight.

Muscle glucose transport

Muscle glucose transport was measured in 24 hr. fasted rats according to the protocol of Dohm *et al.* (15). Ice-cold Krebs Ringer Henseleit Buffer (KHB) containing a lower than normal concentration of $CaCl_2$ (0.62 mM) was saturated with 95% O_2/5% CO_2 prior to the experiment. Soleus muscle strips of approximately 25 mg were isolated using a cutting needle while the tendons remained attached to bone. Muscle strips 1 cm. in length were held at resting length in Plexiglas clips and placed in chambers containing oxygenated KHB and 1% bovine serum albumin (BSA). Following incubation for 60 min at 29°C and with continuous oxygenation, muscle strips were transferred to fresh KHB-BSA medium and incubated an additional 1 hr in the presence of [^{3}H] 2-deoxyglucose [10^6 dpm/ml, 5 mM) and [^{14}C]-sorbitol, 10^5 dpm/ml, 20 mM, ICN Pharmaceuticals, Costa Mesa CA), with or without a maximal concentration of insulin (16,000 µU/ml). Following incubation, the tissue was rinsed, weighed, solubilized and the radioactivity counted. Glucose transport was estimated as the specific uptake of [^{3}H] 2-deoxyglucose. The non-specific component of uptake was estimated using the non-transportable analog [^{14}C]-sorbitol and was subtracted. 2-Deoxyglucose transport was expressed as nmols/g tissue/min.

Statistical analysis

Differences among means were tested using ANOVA or 2-sided, paired student's T test with p = 0.05 defined as the threshold of significance. Values are reported as means $\pm$ SE.

Results

Insulin responsiveness in maturing male Sprague-Dawley rats

Rats aged 6 weeks, 14 weeks, 5 months and 9 months were fasted 24 hours prior to glucose tolerance testing (Fig. 1). Fasting glucose was elevated from 98.9 $\pm$ 5.7 mg/dL at 6 weeks to 113.3 $\pm$ 3.8 at 14 weeks. This elevation was near the threshold of significance (p = 0.057). Compared to 6 weeks, fasting glucose was also elevated at 5 months (140.4 $\pm$ 10.2 mg/dL, p = 0.0092) and at 9 months (140.9 $\pm$ 7.5 mg/dL, p = 0.0023). Fasting insulin was also elevated at 14 weeks (0.84 $\pm$ 0.14 ng/ml) compared to 6 weeks (0.32 $\pm$ 0.06, p = 0.012). Compared to 6 weeks, fasting insulin remained elevated at 5 months (1.29 $\pm$ 0.40 ng/ml, p = 0.0038) and at 9 months (1.18 $\pm$ 0.34 ng/ml, p = 0.0032). The time course of serum glucose concentration following glucose administration was elevated with age (ANOVA, p < 0.01) as was the time course of serum insulin concentration (ANOVA p < 0.01). Specifically, between the ages of 6 and 14 weeks, there were increases in the time course of the concentrations both of glucose (ANOVA, p < 0.05) and of insulin (ANOVA, p < 0.001).

Effect of metformin on insulin responsiveness

Male Sprague-Dawley rats were treated with metformin (320 mg/kg/day) for 20 days. In control rats, food intake increased 34% from 19.4 $\pm$ 1.6 g/24 hr at the beginning of the ex-
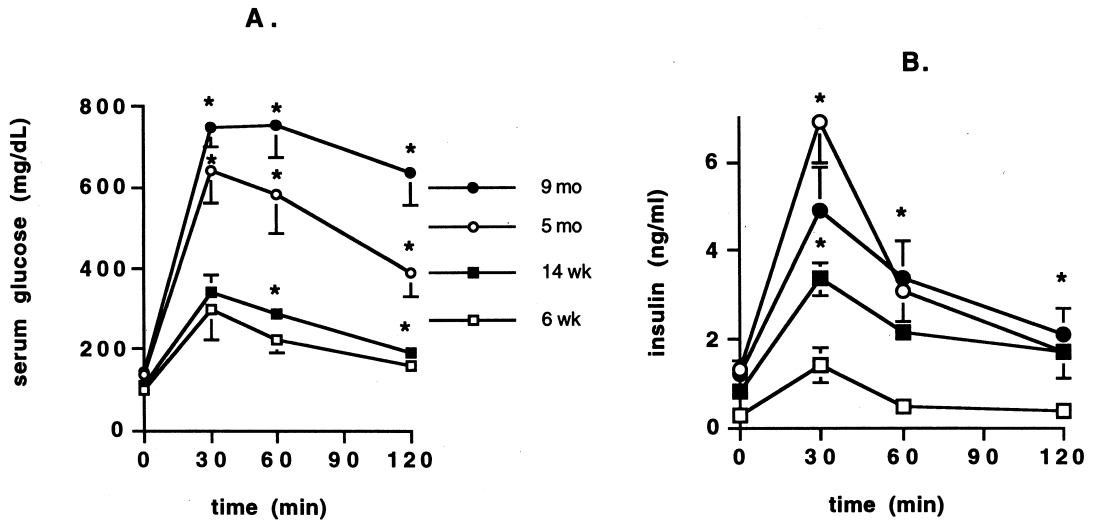
Fig. 1. Glucose tolerance in maturing Sprague-Dawley rats. Rats aged 6 weeks, 14 weeks, 5 months and 9 months were fasted 24 hr. prior to glucose tolerance testing. Initial blood sampling was followed by bolus injection of glucose (3 g/kg, i.p.). Blood samples were obtained at the indicated times. Glucose and insulin were measured as described in Methods. A. Fasting glucose and the profile of the serum glucose following glucose administration were elevated with age. B. Similarly, fasting insulin and the profile of the serum insulin were also elevated with age. * Indicates $p < 0.05$ vs. 6 weeks. Values are means $\pm$ SE, n = 8 for 14 weeks, n = 6 for other ages.

periment to 25.9 $\pm$ 1.3 after 10 days and 26.0 $\pm$ 0.89 at 20 days (Fig. 2). Food intake did not increase in the metformin group and was 19.3 $\pm$ 1.4 at 0 days, 20.0 $\pm$ 1.2 at 10 days (p = 0.0017 vs. control) and 20.7 $\pm$ 0.70 at 20 days (p = 0.0004 vs. control, n = 8). Metformin treatment caused a decrease in adiposity as seen in the reduced wet weight of each of 3 de-
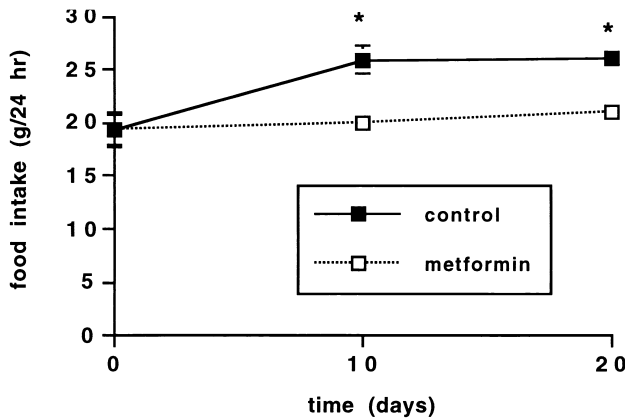

Fig. 2. Metformin prevents increased food intake in male Sprague-Dawley rats. Rats were administered metformin (320 mg/kg/day) in the drinking water. During the course of 20 days of treatment, food intake increased significantly in the control group, but not in the metformin group. * Indicates $p < 0.05$ vs. metformin. Values are means $\pm$ SE, n = 8.

Table 1
Metformin reduces adiposity in male Sprague-Dawley rats. After 20 days for metformin administration, wet weight for various organs was obtained

	Control	Metformin
Initial body weight (g)	303.3 ± 5.9	271.9 ± 7.8*
Final body weight (g)	456.1 ± 11.9	397.1 ± 12.7*
Weight increase (g)	152.9 ± 7.4	125.3 ± 7.3*
Percent weight increase	50.4 ± 2.0	46.2 ± 2.4
PWAT weight (g)	0.62 ± 0.05	0.37 ± 0.04*
RPWAT weight (g)	3.71 ± 0.52	2.63 ± 0.51*
EWAT weight (g)	1.22 ± 0.10	0.79 ± 0.12*
Adiposity index (g/kg)	12.28 ± 1.44	9.58 ± 1.67*
Gastrocnemius weight (g)	2.11 ± 0.15	1.97 ± 0.09
Gastrocnemius glycogen content (mg/g)	0.66 ± 0.06	0.94 ± 0.08*

WAT = white adipose tissue, RWAT = perirenal WAT, RPWAT = retroperitoneal WAT, EWAT = epididymal WAT, lower portion, adiposity index = combined weight of 3 WAT depots/body weight. * Indicates $p < 0.05$. Values are means ± S.E., n = 8.

pots of intra-abdominal white adipose tissue (see Table 1). The combined wet weight of perirenal, retroperitoneal and epididymal fat was 5.56 ± 0.60 g in control rats and 3.79 ± 0.64 g in metformin treated (30 % decrease, p = 0.0011, n =8). These depots were chosen because they are discrete and are labile in response to changes in body weight (16). Metformin also caused a significant 19% decrease in the adiposity index (combined weight of adipose de-
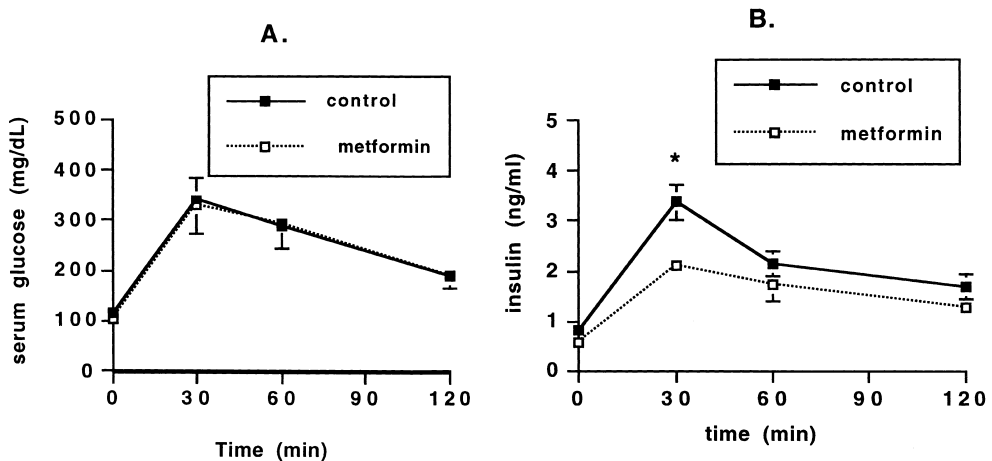

Fig. 3. Metformin reduces insulin secretion during glucose tolerance testing in male Sprague-Dawley rats. Following administration of metformin (320 mg/kg/day) in the drinking water for 20 days, rats were fasted 24 hr. prior to glucose tolerance testing. Initial blood sampling was followed by bolus injection of glucose (3 g/kg, i.p.). Blood samples were obtained at the indicated times. Glucose and insulin were measured as described in Methods. A. The profile of the serum glucose following glucose administration was not affected by metformin treatment. B. The profile of the serum insulin following glucose administration was significantly reduced in metformin-treated rats. * Indicates p < 0.05 vs. metformin. Values are means ± SE, n = 8.

pots/body weight, p = 0.0035, n = 8). The weight of the gastrocnemius muscle was unchanged by metformin treatment (p = 0.413), but gastrocnemius glycogen content was increased 42% (p < 0.013, n = 8). Body weight at the end of the experiment and the increase in body weight were both lower in the metformin group than in controls (see Table 1). However, it should be noted that initial body weight was also lower in the metformin group. Over the course of 20 days, body weight increased 50.4 ± 2.0% in controls and 46.2 ± 2.4% in the metformin group (p = 0.127), indicating that the reduced adiposity had, at most, a slight impact on body weight.

After 20 days of metformin administration, rats were fasted 24 hr and glucose tolerance testing was performed. Blood was sampled for glucose and insulin analysis before i.p. injection of a 3 g/kg glucose load and at 30, 60 and 120 min after. The profiles of serum glucose concentration were virtually identical in control and metformin-treated rats (Fig. 3A). Metformin treatment resulted in a slight lowering of the fasting glucose concentration (8% lower, 113.3 ± 3.76 mg/dL control vs. 104.3 ± 5.5 metformin, p = 0.029, n = 8). Metformin may also have reduced the fasting insulin concentration, although this trend did not reach statistical significance (0.84 ± 0.14 ng/ml control vs. 0.57 ± 0.11 metformin, p = 0.16, n = 8). Metformin treatment significantly reduced the profile of serum insulin concentration following glucose administration (p < 0.01 ANOVA, Fig. 3B). The peak insulin concentration was reduced by 37% (3.37 ± 0.36 ng/ml control vs. 2.11 ± 0.47 metformin, p = 0.017, n = 8).

Following 20 days of metformin treatment, rats were fasted 24 hr and insulin-stimulated glucose transport was measured in isolated strips of soleus muscle (Fig. 4). Basal transport of [^{3}H]-2-deoxyglucose was unchanged by metformin (105.7 ± 11.3 nmol/g/min control vs.
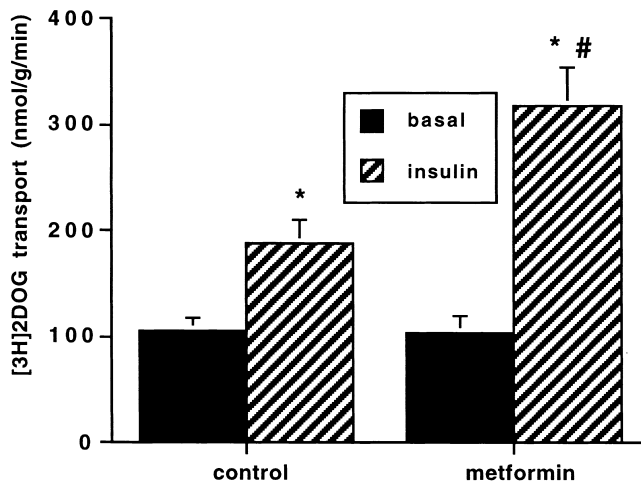

Fig. 4. Metformin enhances *in vitro* glucose transport in muscle strips derived from male Sprague-Dawley rats. Following administration of metformin (320 mg/kg/day) in the drinking water for 20 days, rats were fasted 24 hr. and sacrificed. Strips of soleus muscle were isolated and incubated with [^{3}H]-2-deoxyglucose for 1 hr ± a maximal concentration of insulin as described in Methods. Metformin treatment did not affect basal transport of [^{3}H]-2-deoxyglucose, but significantly elevated insulin-stimulated transport. * Indicates p < 0.05 vs. basal. # Indicates p < 0.01 vs. control. Values are means ± SE, n = 8 for control group and 6 for metformin group.

103.4 $\pm$ 16.4 nmol/g/min metformin, p = 0.907, n = 8 for control, n = 6 for metformin). However, [^{3}H]-2-deoxyglucose transport in response to insulin (16,000 μU/ml) was elevated in the metformin group (187.3 $\pm$ 22.2 nmol/g/min control vs. 316.4 $\pm$ 37.1 nmol/g/min metformin). Insulin stimulated transport 77% over basal in control rats and 206% over basal in metformin treated rats. ANOVA revealed p < 0.01 for the effect of insulin, p < 0.05 for the effect of metformin and p < 0.05 for the interaction.

Discussion

Our findings and those of Goodman *et al*. (8) demonstrate that the male Sprague-Dawley rat aged 14 weeks is a model of reduced insulin responsiveness. We found that between the ages of 6 and 14 weeks, fasting glucose was elevated 15% and fasting insulin elevated 162%. Similarly, glucose tolerance was slightly impaired at 14 compared to 6 weeks, while the profile of serum insulin during glucose tolerance testing was markedly elevated. Goodman *et al*. (8) studied *in vivo* insulin-stimulated glucose uptake in skeletal muscle of Sprague-Dawley males, They reported that both the sensitivity to insulin and the maximum response were reduced between 3 and 8 weeks of age and were further reduced by 16 weeks.

In the present study, 20 days of metformin administration (320 mg/kg/day) caused significant reductions in food intake and in the mass of 3 depots of intra-abdominal white adipose tissue (PWAT, RPWAT and EWAT). Rouru *et al*. (10) found that this same dose of metformin reduced food intake in Zucker rats. The combined mass of these fat depots was reduced 30% in metformin-treated rats. It is not clear whether this represents reversal of adiposity or prevention of an increase that occurred during the course of the experiment. Between the ages of 8 and 14 weeks the combined mass of PWAT, RPWAT and EWAT increases from negligible to 5.56 $\pm$ 0.60 g. In the present study, metformin was present only 20 days out of that period. Thus the figure of a 30% reduction may underestimate the ability of metformin to prevent this increase in adiposity. Although we did not measure lean body mass, the mass of at least one muscle, the gastrocnemius, was not altered by metformin. It is possible that metformin may reduce adiposity without altering lean mass. Metformin, had only a small effect on the increase in body weight that occurred over the course of 20 days, an effect which did not reach the level of statistical significance (see Table 1). This is not surprising, since it is likely that both lean mass and subcutaneous fat mass increased during the experiment.

Metformin caused a significant reduction in the profile of serum insulin following administration of a glucose load. This most likely represents an increased sensitivity to insulin in terms of stimulating the rate of whole-body glucose disposal. In order to determine whether muscle is a major site for increased glucose disposal, we examined the effect of metformin treatment on a glucose transport in an *in vitro* soleus muscle preparation. We found that metformin treatment had no effect on basal glucose transport, but elevated, by almost 3 fold, the increase in transport occurring in response to a maximal concentration of insulin.

Metformin has long been known to enhance whole-body glucose disposal. Although adipose tissue and skeletal muscle are the most likely candidates as sites for increased glucose transport, neither has been clearly implicated. Pedersen *et al*. (18) studied adipocytes of obese insulin-resistant patients and found that metformin therapy had no effect on insulin-stimulated glucose transport or on insulin binding. On the other hand, Matthai *et al*. (9) studied

adipocytes obtained from (fa/fa) Zucker rats and found that metformin increased insulin-stimulation of glucose transport and of GLUT4 translocation.

Similarly, there have been mixed reports regarding the effect of metformin on muscle glucose transport. Galuska *et al.* (5) studied muscle strips derived from insulin-resistant human subjects and found that *in vitro* treatment with metformin, enhances insulin-stimulated transport of non-hydrolyzable glucose analogs. However, this enhancement occurs only under euglycemic conditions and not under the hyperglycemic conditions which are present in these subjects. Moreover, the concentration of metformin required is 10-fold higher than a typical clinical concentration (19). One problem with this study is that short-term *in vitro* administration of metformin may not reproduce the effects of longer-term *in vivo* treatment. The clinical effects of metformin may require either a systemic mediator or a longer period of exposure. Hundal *et al.* (20) reported that treatment with metformin *in vitro* increases glucose transport in cultured muscle cells, but that this enhancement is insulin-independent. Animal studies to date have failed to show that chronic metformin administration enhances insulin-responsiveness in skeletal muscle. Kemmer *et al.* (6) administered metformin (250 mg/kg/day) to non-diabetic rats which had been placed on a restricted diet. They found no effect of metformin on insulin-stimulated glucose transport in an isolated perfused muscle preparation. Bailey *et al.* (21) reported that in mouse soleus muscle of streptozotocin-treated rats, metformin produced only a very modest enhancement of insulin-stimulated 3-O-methylglucose transport. Only one study to date has examined the effect of metformin treatment on skeletal muscle of insulin-resistant rats. Rouru *et al.* (7) treated obese Zucker rats with metformin (300 mg/kg/day) for 12 days and found no change in insulin-stimulated [^{3}H] 2-deoxyglucose transport is isolated strips of tibialis anterior muscle. In contrast, we found that in Sprague-Dawley rats treated with metformin for 20 days, there was a marked enhancement of insulin-stimulated [^{3}H] 2-deoxyglucose transport in soleus muscle strips. The difference in our results may stem from the use of a different strain of rats or from the use of an oxidative muscle (soleus) as opposed to a glycolytic muscle (tibialis anterior). Alternatively, the difference may result from the methodology employed. We found that saturating KHB buffer with oxygen when it is ice-cold and kept under pressure results in greater oxygen content and elevates [^{3}H] 2-deoxyglucose and insulin responsiveness. This may explain why we obtained much higher rates of [^{3}H] 2-deoxyglucose transport and greater insulin-responsiveness than did Rouru *et al.* (7). Our report is the first to demonstrate that chronic metformin administration to animals with reduced insulin responsiveness enhances insulin-stimulated glucose transport in skeletal muscle.

Potential sites of metformin action in muscle include the insulin receptor, insulin signal transduction, the GLUT4 transporter and GLUT4 translocation to the cell surface. There are conflicting reports as to whether metformin increases the density of muscle insulin receptors (21, 22). However, both these studies were performed with streptozotocin-treated rodents, and do not address the more important question on the effect of metformin on insulin-resistant muscle. Rossetti *et al.* (23) reported that metformin increases insulin-stimulated tyrosine kinase activity in muscle. Handberg *et al.* (24) and Klip *et al.* (23) have each shown that metformin does not increase muscle expression of the insulin-sensitive transporter, GLUT4. However, metformin may enhance insulin-stimulated translocation of GLUT4 without affecting its expression.

Evidence now exists that metformin may directly enhance glucose utilization in the form of both glucose oxidation and glycogen synthesis (2). Bailey *et al.* (21) reported that metformin increases hexokinase expression, which may in turn increase intracellular oxidation. Reddi *et al.* (13) reported that in the KK mouse model of insulin-resistance, metformin increase muscle glycogen stores and glycogen synthase activity. We also found that metformin increased glycogen stores in gastrocnemius muscle. Further studies will be needed to explain the mechanism by which metformin enhances glucose transport in insulin-resistant muscle. Further studies will also be required to determine whether metformin affects post-transport glucose metabolism.

Acknowledgments

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