Nitric Oxide Increases Glucose Uptake Through a Mechanism That Is Distinct From the Insulin and Contraction Pathways in Rat Skeletal Muscle

Yasuki Higaki, Michael F. Hirshman, Nobuharu Fujii, and Laurie J. Goodyear

Insulin, contraction, and the nitric oxide (NO) donor, sodium nitroprusside (SNP), all increase glucose transport in skeletal muscle. Some reports suggest that NO is a critical mediator of insulin- and/or contraction-stimulated transport. To determine if the mechanism leading to NO-stimulated glucose uptake is similar to the insulin- or contraction-dependent signaling pathways, isolated soleus and extensor digitorum longus (EDL) muscles from rats were treated with various combinations of SNP (maximum 10 mmol/l), insulin (maximum 50 mU/ml), electrical stimulation to produce contractions (maximum 10 min), wortmannin (100 nmol/l), and/or the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) (0.1 mmol/l). The combinations of SNP plus insulin and SNP plus contraction both had fully additive effects on 2-deoxyglucose uptake. Wortmannin completely inhibited insulin-stimulated glucose transport and only slightly inhibited SNP-stimulated 2-deoxyglucose uptake, whereas L-NMMA did not inhibit contraction-stimulated 2-deoxyglucose uptake. SNP significantly increased the activity of the α1 catalytic subunit of 5′AMP-activated protein kinase (AMPK), a signaling molecule that has been implicated in mediating glucose transport in fuel-depleted cells. Addition of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (1 mg/ml) to the drinking water of rats for 2 days failed to affect the increase in muscle 2-deoxyglucose uptake in response to treadmill exercise. These data suggest that NO stimulates glucose uptake through a mechanism that is distinct from both the insulin and contraction signaling pathways.
***Diabetes* 50:241–247, 2001**

Nitric oxide (NO) is produced in a variety of tissues; this occurs through the activation of different isoforms of NO synthase (NOS) (1). Of the three members of the NOS family that have been identified, neuronal NOS (nNOS) and endothelial NOS (eNOS) are expressed in skeletal muscle (2). It has been reported that NO is released from isolated extensor digitorum longus (EDL) muscles incubated at rest and that prior electrical stimulation used to generate contractile activity in these muscles further increases NO release (3). Treadmill exercise can also activate NOS in gastrocnemius muscles (4), providing additional evidence that NO production in skeletal muscle increases during exercise. During the last few years, it has been proposed that NO mediates exercise-stimulated glucose transport in skeletal muscle (5,6). Exogenously administered NO, which is generated from the NO donor, sodium nitroprusside (SNP), stimulates glucose transport in isolated skeletal muscles (6–8) by increasing GLUT4 levels at the cell surface (8). In studies in which rats were first exercised on a treadmill (5) or had hindlimb muscles contracted via nerve stimulation (6) followed by isolation of muscles and measurement of glucose transport, data have shown that NOS inhibition blocked exercise/contraction-stimulated glucose transport. In contrast, another study has shown that when muscles are isolated and then contracted in vitro in the presence of a NOS inhibitor, there is normal activation of contraction-stimulated glucose transport (8), suggesting that there are differences between in vivo and in vitro muscle stimulation.

Insulin is a potent stimulator of glucose transport in skeletal muscle. Part of the mechanism by which insulin increases glucose transport in vivo involves enhanced blood flow and glucose delivery to the muscle, a process mediated by the release of NO from the endothelium (9–11). The acute administration of the NOS inhibitors N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) results in the development of marked insulin resistance, hypertension, and/or hyperglycemia (9,12,13). NOS blockade decreases blood flow to skeletal muscle and impairs insulin-mediated glucose disposal during a hyperinsulinemic-euglycemic clamp in vivo (9,12). In contrast to the effects of NOS inhibition in vivo, NOS inhibitors fail to affect insulin-stimulated glucose transport in isolated muscles incubated using in vitro preparations (6,8,9); this suggests that hemodynamic factors are needed to fully amplify the increase in insulin-stimulated glucose transport in skeletal muscle.

5′AMP-activated protein kinase (AMPK) has recently emerged as a putative regulator of multiple metabolic processes in skeletal muscle, including fatty acid and carbohydrate metabolism (14–17). We (18,19) and others (20,21) have provided evidence that AMPK is an intermediary in the signaling cascade leading to contraction-stimulated glucose transport in skeletal muscle. AMPK is a heterotrimer consisting of three subunits, α, β, and γ. The α subunit contains

From the Research Division, Joslin Diabetes Center; Department of Medicine, Brigham and Women's Hospital; and Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Laurie J. Goodyear, PhD, Research Division, Joslin Diabetes Center, 1 Joslin Place, Boston, MA 02215. E-mail: laurie.goodyear@joslin.harvard.edu.

Received for publication 4 February 2000 and accepted in revised form 24 October 2000.

AMPK, AMP-activated protein kinase; EDL, extensor digitorum longus; eNOS, endothelial nitric oxide synthase; KRBB, Krebs-Ringer bicarbonate buffer; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NOS; PI3K, phosphatidylinositol 3-kinase; SNP, sodium nitroprusside.

the kinase domain and contributes to the ATP-binding site (22). Of the two isoforms of the α subunits, α1 AMPK is widely expressed in liver, pancreas, adipose tissue, and skeletal muscle, whereas α2 AMPK catalytic isoform is predominantly expressed in skeletal muscle (23,24). Interestingly, AMPK coimmunoprecipitates with eNOS in rat cardiac muscle, and presently there are data showing that AMPK can phosphorylate eNOS in an in vitro assay (25). However, it is unknown whether there is an interaction between NO and AMPK signaling to stimulate glucose transport in skeletal muscle.

In the current study, we determined if the signaling mechanism leading to NO-stimulated glucose transport is similar to, or distinct from, the signaling mechanisms leading to insulin- and contraction-stimulated glucose transport in rat skeletal muscle. Our results demonstrate that NO signaling to glucose transport is independent of the mechanisms through which insulin and muscle contraction increase transport. Furthermore, our data demonstrate that NO-stimulated glucose uptake is associated with an activation of the α1 catalytic subunit of AMPK.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing ~40 g were purchased from Taconic Farms. Animals were housed in an animal room maintained at 23°C with a 12-h light/dark cycle and fed standard laboratory diet and water ad libitum. All protocols for animal use and euthanasia were reviewed and approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and accorded with NIH guidelines.

Materials. SNP, wortmannin, L-NAME, LY-83583, D-glucose, mannitol, and pyruvic acid were purchased from Sigma Chemical (St. Louis, MO). L-NMMA was purchased from Calbiochem (San Diego, CA), and 2-deoxy-D-[1,2-^{3}H]glucose and D-[^{14}C]mannitol were purchased from New England Nuclear (Boston, MA).

Muscle incubations. Rats were fed ad libitum before muscle isolation, and experiments commenced between 9:00 and 10:00 A.M.. Animals were killed by decapitation, and the EDL muscles were rapidly dissected. Both ends of each muscle were tied with suture (silk 4–0) and mounted on an incubation apparatus as previously described (18). The buffers were continuously gassed with 95% O_2:5% CO_2. Muscles were preincubated in 6 ml Krebs-Ringer bicarbonate buffer (KRBB) containing 2 mmol/l pyruvate at 37°C for 50 min in the presence or absence of SNP (1, 5, 10, or 20 mmol/l) or insulin (50 mU/ml). For contraction treatment, muscles were stimulated during the last 10 min of the 50-min incubation period (train rate 2/min, train duration 10 s, rate 100 pulses/s, duration 0.1 ms, 100 V). When added, the inhibitors wortmannin (100 nmol/l), LY-83583 (10 μmol/l), and L-NMMA (0.1 mmol/l) were present throughout the entire incubation and present ≥30 min before stimulation. In preliminary experiments, dose-dependent inhibition of L-NMMA on NOS activity was measured in EDL muscle using the method previously described by Roy et al. (9). Altogether, 1 μmol/l L-NMMA inhibited NOS activity ~50%, 10 μmol/l inhibited NOS activity ~85%, and 0.1 mmol/l L-NMMA (the dose we used in the current experiments) inhibited NOS activity ~90%. Then, muscles were immediately used for the measurement of 2-deoxyglucose uptake or were immediately frozen in liquid nitrogen and subsequently analyzed for the measurement of ATP, creatine phosphate, and glycogen concentrations and isoform-specific AMPK activity.

2-Deoxyglucose uptake. The 2-deoxyglucose uptake was measured in 2 ml KRBB containing 1 mmol/l 2-deoxy-D[1,2-^{3}H]glucose (1.5 μCi/ml) and 7 mmol/l D-[^{14}C]mannitol (0.45 μCi/ml) (New England Nuclear, Boston, MA) at 30°C for 10 min. SNP, insulin, wortmannin, LY83583, and L-NMMA were added to the buffer if they were present during the previous incubation period. Muscles were processed, radioactivity was determined by liquid scintillation counting for dual labels, and 2-deoxyglucose uptake was calculated as previously described (26).

Assays for muscle enzymes and metabolites. To measure ATP and creatine phosphate concentrations, frozen muscles were homogenized in $HClO_4$ in an ethanol-solid CO_2 bath and centrifuged at 14,000*g* for 10 min at –5°C. The supernatant of the homogenates was neutralized with a solution of 2N KOH, 0.4 mol/l imidazole, and 0.4 mol/l KCl and then centrifuged at 14,000*g* at –9°C. The supernatant was collected and analyzed enzymatically for ATP and creatine phosphate (27).

For measurement of muscle glycogen, muscles were dissolved in 30% KOH and 5% Na_2SO_4 at 70°C for 15 min. Glycogen was then precipitated by mixing with 3 × volume of absolute alcohol and stored overnight at –20°C. The precipitates were collected by centrifugation at 13,000*g* for 5 min. The glycogen was hydrolyzed in 6N H_2SO_4 at 100°C for 45 min and cooled. Samples were neutralized with 1N NaOH and glucose was measured using the glucose (HK) reagent (Sigma Chemical).

For the measurement of isoform-specific AMPK activity, muscles were homogenized in ice-cold lysis buffer (1:100, wt/vol) containing 20 mmol/l Tris-HCl (pH 7.4), 1% Triton X-100, 50 mmol/l NaCl, 250 mmol/l sucrose, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, 2 mmol/l dithiothreitol, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mmol/l benzamidine, and 0.5 mmol/l phenylmethylsulfonyl fluoride, and centrifuged at 14,000*g* for 20 min at 4°C. The supernatants (200 μg protein) were immunoprecipitated with isoform-specific antibodies to the α1 or α2 catalytic subunits of AMPK and protein A/G beads. These are anti-peptide antibodies made to the amino acid sequences DFYLATSPPDSFLDDHHLTR (339–358) of α1 and MDDSAMHIPPGLKPH (352–366) of α2. Immnoprecipitates were washed twice in both lysis buffer and in wash buffer (240 mmol/l HEPES and 480 mmol/l NaCl). Kinase reactions were performed in 40 mmol/l HEPES (pH 7.0), 0.2 mmol/l SAMS peptide (synthetic substrate for AMPK), 0.2 mmol/l AMP, 80 mmol/l NaCl, 0.8 mmol/l dithiothreitol, 5 mmol/l $MgCl_2$, 0.2 mmol/l ATP (2 μCi [γ-^{32}P]ATP), and in a final volume of 40 μl for 20 min at 30°C (28). At the end of the reaction, a 20-μl aliquot was removed and spotted on Whatman P81 paper. The papers were washed six times in 1% phosphoric acid and once with acetone. ^{32}P incorporation was quantitated with a scintillation counter, and kinase activity was expressed as fold increases compared with basal samples.

In situ contraction studies. Rats were fed ad libitum before muscle isolation. Animals were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body wt). The sciatic nerve was dissected free and connected to subminiature electrodes (Harvard Apparatus, South Natick, MA). A pin under the patella tendon fixed the knee of the leg, and a 100-g weight was attached by a wire around the Achilles' tendon. The sciatic nerve was stimulated for two 5-min periods (train rate 1/s, train duration 500 ms, rate 100 pulses/s, duration 0.1 ms, 3–10 V) separated by 1 min of rest. Immediately after stimulation, EDL muscles were dissected and incubated in 3 ml KRBB containing 8 mmol/l mannitol for 20 min in the absence or presence of L-NMMA. After this incubation period, 2-deoxyglucose uptake was measured as previously described.

Treadmill exercise studies in L-NAME–treated animals. Rats were accustomed to a rodent treadmill (Quinton Instruments, Seattle, WA) for 5 min/day for 2 days before the experiment. L-NAME was added to the drinking water (1 mg/ml), and the water was changed daily for 2 days before the experiment. Systolic blood pressure was measured by a volume-oscillometric method (UR-5000; Ueda, Tokyo) before and during the 2–day L-NAME treatment. Rats ran on a rodent treadmill with a 10% incline for 1 h at 0.7 mph. Animals were killed immediately after exercise, and both the soleus and EDL muscles were rapidly dissected and mounted on the incubation apparatus. The muscles were incubated for 20 min in KRBB containing 8 mmol/l D-mannitol at 30°C, and 2-deoxyglucose uptake was measured as previously described.

Statistical analysis. Data are means ± SE. The effect of SNP on 2-deoxyglucose uptake, ATP, creatine phosphate, and isoform-specific AMPK activity was compared by a one-way analysis of variance with Fisher's protected least significant difference test. For comparison of two means, an unpaired Student's *t* test was performed.

RESULTS

Effect of SNP on glucose uptake in isolated EDL muscles. SNP resulted in a dose-dependent increase in 2-deoxyglucose uptake in isolated EDL muscles (Fig. 1). The maximal increase was twofold over basal levels at a concentration of 10 mmol/l SNP and did not increase further with 20 mmol/l SNP.

SNP stimulates glucose uptake through an insulin-independent pathway in isolated EDL muscles. To determine whether the combination of SNP and a maximally effective dose of insulin has additive effects on skeletal muscle glucose uptake, isolated EDL muscles were incubated in KRBB in the absence or presence of insulin and/or 10 mmol/l SNP. Figure 2*A* shows that the combination of SNP and insulin had nearly full additive effects on 2-deoxyglucose uptake. To determine whether SNP and insulin stimulate glucose uptake by different signaling mechanisms, isolated

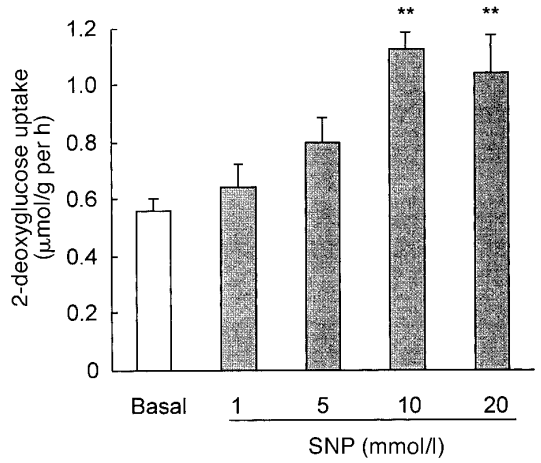

FIG. 1. SNP-stimulated glucose uptake in isolated EDL muscle. 2-Deoxyglucose uptake was measured in the absence or presence of 1, 5, 10, and 20 mmol/l SNP as described in RESEARCH DESIGN AND METHODS. Data are means ± SE. **P< 0.01 vs. basal; n = 4–20 per group.

muscles were incubated in the presence or absence of the phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin (100 nmol/l), before stimulation and throughout the remainder of the study (Fig. 3). As we (18,19) and others (29,30) have previously observed, wortmannin completely inhibited insulin-stimulated glucose uptake and had no effect on contraction-stimulated uptake. In contrast, wortmannin only partially (~35%) decreased SNP-stimulated glucose uptake. These findings suggest that at least part of the mechanism by which SNP and insulin stimulate glucose uptake is distinct.

Additive effect of SNP plus contraction on glucose uptake in isolated EDL muscles. Next, we investigated whether SNP and contraction stimulate glucose uptake through the same mechanisms by determining if the combination of SNP and contraction has an additive effect on glucose uptake. Isolated EDL muscles were incubated in KRBB in the absence or presence of 10 mmol/l SNP, with or without electrical stimulation for muscle contraction. Interestingly, the combination of SNP and contraction had fully additive effects on skeletal muscle glucose uptake (Fig. 2*B*), suggesting different mechanisms for SNP- and contraction-stimulated glucose uptake.

To test this hypothesis further, we used the guanylate cyclase inhibitor LY-83583 that inhibits SNP-stimulated 2-deoxyglucose uptake in isolated muscles, as previously reported (7). Consequently, 10 μmol/l LY-83583 blocked SNP-stimulated 2-deoxyglucose uptake in the isolated EDL muscles, whereas the inhibitor did not block contraction-stimulated 2-deoxyglucose uptake (data not shown). However, we found that LY-83583 had nonspecific effects on skeletal muscle glucose uptake. In the presence of a low dose of the inhibitor (10 μmol/l), basal 2-deoxyglucose uptake increased twofold compared with the vehicle. In contrast, higher doses of the inhibitor (~50–250 μmol/l) induced muscle stiffness and dose-dependently decreased basal rates of 2-deoxyglucose uptake. LY-83483 at 10 μmol/l had no effect on the development of tension during contraction, whereas higher concentrations altered resting tension and significantly impaired the ability to develop tension during contraction (data not shown). Therefore, we do not believe that LY-83583 can be

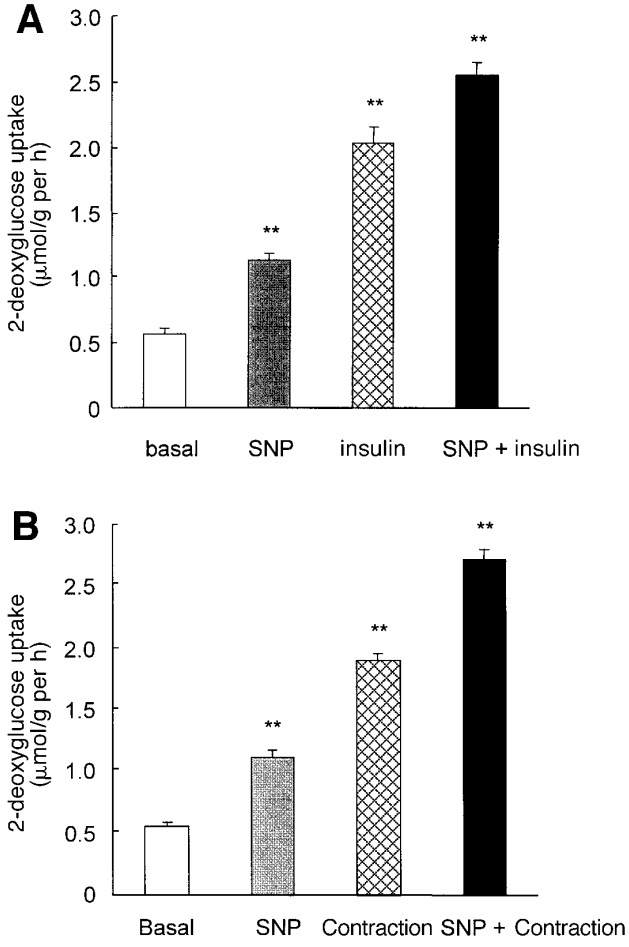

FIG. 2. Effects of SNP on insulin-stimulated 2-deoxyglucose uptake (*A*) and effect of SNP on contraction-stimulated 2-deoxyglucose uptake (*B*) in isolated EDL muscles. Data are means ± SE. **P< 0.01 vs. basal condition; n = 6–20 per group.

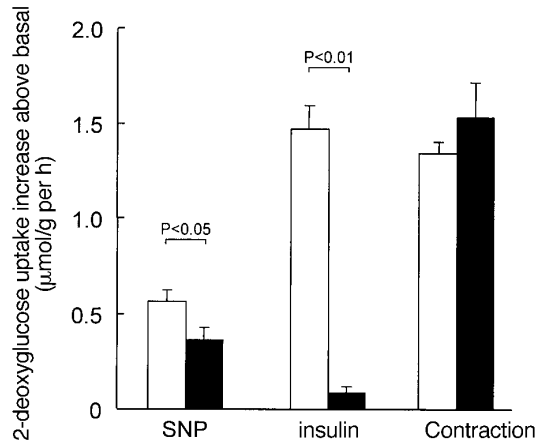

FIG. 3. Effects of wortmannin (100 nmol/l) (■) on SNP-, insulin-, or contraction-stimulated 2-deoxyglucose uptake in isolated EDL muscles. 2-Deoxyglucose uptake was measured in the absence or presence of 10 mmol/l SNP as described in RESEARCH DESIGN AND METHODS. Data are means ± SE; n = 3–20 per group.

TABLE 1
Effect of SNP on ATP, creatine phosphate, and glycogen concentrations in isolated EDL muscle

	SNP (mmol/l)			
	Basal	1	5	10
ATP (nmol/mg)	5.9 ± 0.2	5.9 ± 0.3	6.3 ± 0.2	6.2 ± 0.2
Creatine phosphate (nmol/mg)	15.3 ± 0.9	14.0 ± 1.3	14.9 ± 0.5	17.2 ± 1.7
Glycogen (nmol/mg)	29.5 ± 1.8	28.4 ± 2.3	31.5 ± 0.9	30.9 ± 1.4

Data are means ± SE; n = 5 per group.

used to determine the role of NO in the regulation of contraction-stimulated glucose uptake in isolated muscles.

Effect of SNP on ATP, creatine phosphate, and glycogen concentrations and α1 and α2 AMPK activities in isolated EDL muscles. One report suggests that high concentrations of SNP decrease ATP and creatine phosphate concentrations in EDL muscles (6). We hypothesized that under these conditions, SNP may also increase AMPK activity, which is activated in response to cellular fuel depletion. SNP treatment of the muscles at concentrations ranging from 1–10 mmol/l did not alter ATP, creatine phosphate, or glycogen concentrations (Table 1). In contrast to the lack of effect of SNP on these muscle metabolites, SNP significantly increased α1 AMPK activity. Interestingly, SNP had no effect on α2 AMPK activity, whereas the contraction-stimulated increase in both α1 and α2 AMPK activity was significantly greater than that observed with SNP (Fig. 4). These data suggest distinct regulatory mechanisms leading to an increase in isoform-specific AMPK activity in rat skeletal muscle.

Effect of L-NMMA on 2-deoxyglucose uptake. To determine whether the NOS inhibitor L-NMMA affects basal- and contraction-stimulated glucose uptake, we first used isolated soleus and EDL muscles that were preincubated and contracted in KRBB in the absence or presence of 0.1 mmol/l L-NMMA. Contraction increased 2-deoxyglucose uptake 2.9-fold above basal in the soleus muscles and 3.8-fold above basal in the EDL muscles. L-NMMA treatment in vitro had no effect on basal or contraction–stimulated 2-deoxyglucose uptake in either the soleus or EDL muscles (Fig. 5).

Then, we contracted (or sham treated) EDL muscles in situ via sciatic nerve stimulation, removed the muscles, and incubated them in KRBB in the absence or presence of 0.1 mmol/l L-NMMA. As shown in Fig. 6, and similar to the results obtained in vitro, L-NMMA had no effect on basal- or contraction-stimulated 2-deoxyglucose uptake in the EDL muscles.

Effect of treadmill running on 2-deoxyglucose uptake in L-NAME–treated animals. The administration of L-NAME via the drinking water for 2 days resulted in a significant increase in systolic blood pressure from 104 ± 2 mmHg before L-NAME treatment to 132 ± 3 mmHg after 2 days of treatment (P < 0.001). L-NAME treatment had no effect on exercise tolerance, as all animals were able to complete the 60-min exercise task. Isolated soleus muscles from L-NAME–treated rats had lower basal 2-deoxyglucose uptake compared with untreated animals (Fig. 7). In contrast, 2 days of L-NAME treatment did not affect exercise–stimulated 2-deoxyglucose uptake in the soleus muscles.

DISCUSSION

There are at least two distinct signaling cascades that stimulate glucose transport in muscle. One pathway is stimulated by insulin and insulin-like growth factor-1; based on studies using wortmannin and LY294002, PI3K is necessary for activation of glucose transport by this mechanism (29,30). Another insulin-independent pathway, often referred to as the contraction or contraction/hypoxia pathway is wortmannin insensitive and apparently PI3K-independent (31–34). The combination of insulin and contraction have additive or partially additive effects on glucose transport; this supports the hypothesis that separate signaling pathways regulate insulin- and contraction-stimulated glucose uptake. In the current study, we observed additive effects of SNP plus insulin on skeletal muscle glucose transport and found that NO-stimulated transport is only partially wortmannin inhibitable. Altogether, these findings demonstrate that the NO signaling pathway mediates skeletal muscle glucose uptake through an insulin-independent pathway. This finding is consistent with the observations of several other studies (6,8,9,35).

The administration of L-NMMA in vivo results in the development of marked insulin resistance (9,12,13) and hypergly-

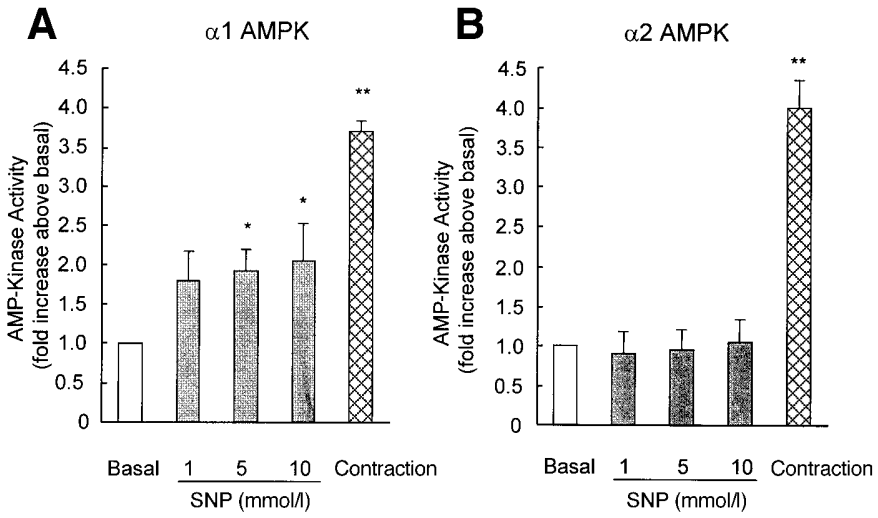

FIG. 4. Effect of SNP or contraction on α1 AMPK activity (*A*) and α2 AMPK activity (*B*) in isolated EDL muscles. Data are means ± SE; **P* < 0.05, ***P* < 0.01 vs. basal condition; *n* = 5–9 per group.

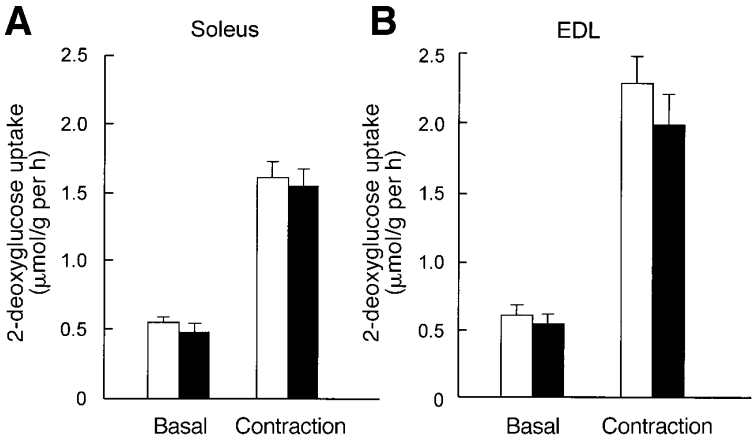

FIG. 5. In vitro contraction-stimulated 2-deoxyglucose uptake in the presence (■) or absence of L-NMMA in isolated soleus muscles (*A*) and EDL (*B*) muscles. Data are means ± SE; *n* = 5–8 per group.

cemia (13), suggesting an important role for NO in muscle glucose metabolism. Interestingly, these effects of NOS inhibition on insulin-mediated glucose uptake in vivo are not observed when isolated skeletal muscles are incubated with NOS inhibitors and insulin in vitro (6,8,9,35). These findings, combined with our results showing additive effects of SNP plus insulin and the partial inhibition using wortmannin, imply that NO augments insulin's effects systemically, most likely not at the level of skeletal muscle fibers.

Previous studies assessing the role of NO in exercise/contraction–stimulated glucose uptake have resulted in contrasting conclusions (5,6,8). In one report, hindlimb muscles were contracted in situ via electrical stimulation of the sciatic nerve, and the EDL muscles were isolated and used for measurement of glucose transport in the presence or absence of the NOS inhibitor L-NMMA (6). Under these conditions, the NOS inhibitor was shown to fully block contraction-stimulated glucose transport. In addition, another report showed that blocking NOS by adding L-NAME to the drinking water of rats prevented GLUT4 translocation to skeletal muscle sarcolemma and glucose transport in response to acute exercise (5). In contrast, when epitrochlearis muscles were contracted in vitro in the absence or presence of L-NMMA and glucose transport was measured, NOS inhibition had no effect on glucose transport (8). In the current study, three independent experiments were performed to assess this problem: *1*) hindlimb muscles were contracted in vivo via electrical stimulation of the sciatic nerve, and then isolated EDL muscles were used for measurement of glucose uptake in the presence or absence of L-NMMA (similar to the methods of Balon and Nadler [6]); *2*) isolated incubated EDL muscles were used for measurement of contraction-stimulated glucose uptake in the presence or absence of L-NMMA (similar to the methods of Etgen et al. [8]); and *3*) L-NAME–treated rats performed running exercise for 1 h, and isolated soleus muscles were used for measurement of glucose uptake. For all three experiments, the NOS inhibitors failed to affect exercise or contraction-stimulated 2-deoxyglucose uptake. Furthermore, the combination of contraction and SNP had additive effects on glucose uptake. Therefore, our data are consistent with the conclusion that NO is not involved in the signaling pathway leading to contraction-stimulated glucose uptake in skeletal muscle.

We (18,19) and others (20,21) have hypothesized that AMPK is an essential intermediary in the signaling cascade leading to contraction-stimulated glucose transport in skeletal muscle. AMPK activity is increased when cells sense low

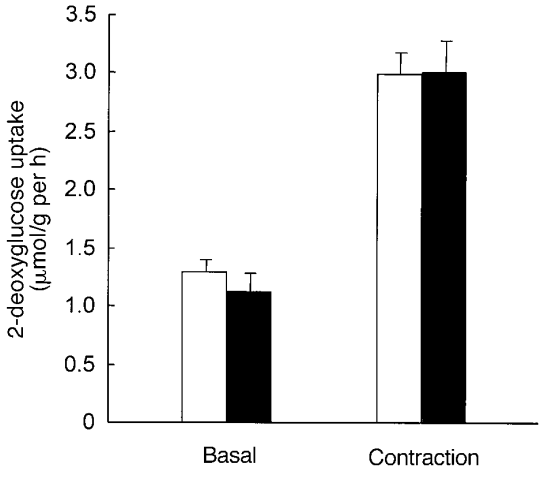

FIG. 6. In situ contraction-stimulated 2-deoxyglucose uptake. Contracted EDL muscles were dissected and incubated in the presence (■) or absence of L-NMMA in vitro, and then 2-deoxyglucose uptake was measured. Data are means ± SE; *n* = 8–11 per group.

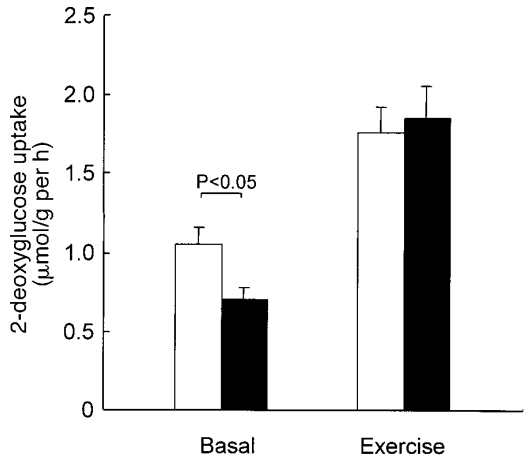

FIG. 7. Effect of 1-h treadmill exercise on 2-deoxyglucose uptake in isolated soleus muscle. L-NAME (■) was added to the drinking water of rats for 2 days before the experiment. Data are means ± SE; *n* = 6 per group.

fuel, acting as a "fuel gauge" and functioning to increase ATP generation under conditions of increased energy expenditure (19,36). Because one report suggested that incubation of skeletal muscle with a high concentration of SNP induces a significant decrease in ATP and creatine phosphate concentrations (6), we hypothesized that SNP would increase AMPK activity in skeletal muscle and that this might be part of the mechanism leading to the activation of glucose uptake. The α2 catalytic subunit of AMPK is highly expressed in skeletal muscle (23,24), and in contrast to electrical stimulation, which activates both α1 and α2 isoforms, only the α2 isoform has been reported to be increased by physical exercise in rats and humans (37,38). Surprisingly, a wide range of SNP concentrations did not significantly alter ATP, creatine phosphate, or glycogen concentrations in the incubated muscles, but these results were consistent with the lack of activation of the α2 catalytic subunit of AMPK. The lack of α2 AMPK activation with SNP is also consistent with distinct signaling mechanisms leading to contraction- and NO-stimulated glucose uptake.

Despite the lack of α2 activation and the lack of changes in ATP and creatine phosphate with SNP treatment, α1 AMPK activity was significantly increased in the incubated EDL muscles. These observations suggest that there are distinct mechanisms for the regulation of α1 and α2 AMPK activity in skeletal muscle. Furthermore, these observations demonstrate that these two catalytic isoforms may have different sensitivities to ATP and creatine phosphate in the intact muscle. In future studies, it will be interesting to determine if cytokines activate α1 AMPK in skeletal muscle, because cytokines are thought to modulate muscle glucose transport by increasing NO production (39).

In summary, our NOS-inhibitor data suggest that NO is not involved in the signaling pathway leading to contraction-stimulated glucose uptake in skeletal muscle and that SNP increases skeletal muscle glucose uptake through a mechanism that is distinct from the insulin- and contraction-signaling pathways. These observations suggest that there is a third signaling pathway that enhances glucose uptake in skeletal muscle. Furthermore, our data demonstrate that NO-stimulated glucose uptake is associated with an activation of the α1 catalytic subunit of AMPK.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Arthritis and Musculoskeletal and Skin Diseases Grants AR-45670 and AR-42238 (L.J.G.). Y.H. was supported by the Ministry of Education, Science, Sports, and Culture of Japan. N.F. was supported by a postdoctoral fellowship for research abroad from Japan Society for the Promotion of Science.

REFERENCES

1. Moncada S, Higgs A: The L-arginine-nitric oxide pathway. *N Engl J Med* 329:2002–2012, 1993
2. Kobzik L, Reid MB, Bredt DS, Stamler JS: Nitric oxide in skeletal muscle. *Nature* 372:546–548, 1994
3. Balon TW, Nadler JL: Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol* 77:2519–2521, 1994
4. Roberts CK, Barnard RJ, Jasman A, Balon TW: Acute exercise increases nitric oxide synthase activity in skeletal muscle. *Am J Physiol* 277:E390–E394, 1999
5. Roberts CK, Barnard RJ, Scheck SH, Balon TW: Exercise-stimulated glucose transport in skeletal muscle is nitric oxide dependent. *Am J Physiol* 273:E220–E225, 1997
6. Balon TW, Nadler JL: Evidence that nitric oxide increases glucose transport in skeletal muscle. *J Appl Physiol* 82:359–363, 1997
7. Young ME, Radda GK, Leighton B: Nitric oxide stimulates glucose transport and metabolism in rat skeletal muscle in vitro. *Biochem J* 322:223–228, 1997
8. Etgen GJ Jr, Fryburg DA, Gibbs EM: Nitric oxide stimulates skeletal muscle glucose transport through a calcium/contraction- and phosphatidylinositol-3-kinase-independent pathway. *Diabetes* 46:1915–1919, 1997
9. Roy D, Perreault M, Marette A: Insulin stimulation of glucose uptake in skeletal muscles and adipose tissues in vivo is NO dependent. *Am J Physiol* 274: E692–E699, 1998
10. Baron AD, Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G: Insulin-mediated skeletal muscle vasodilation contributes to both insulin sensitivity and responsiveness in lean humans. *J Clin Invest* 96:786–792, 1995
11. Baron AD: The coupling of glucose metabolism and perfusion in human skeletal muscle: the potential role of endothelium-derived nitric oxide. *Diabetes* 45 (Suppl. 1):S105–S109, 1996
12. Baron AD, Zhu JS, Marshall S, Irsula O, Brechtel G, Keech C: Insulin resistance after hypertension induced by the nitric oxide synthesis inhibitor L-NMMA in rats. *Am J Physiol* 269:E709–E715, 1995
13. Shankar R, Zhu JS, Ladd B, Henry D, Shen HQ, Baron AD: Central nervous system nitric oxide synthase activity regulates insulin secretion and insulin action. *J Clin Invest* 102:1403–1412, 1998
14. Kemp BE, Mitchelhill KI, Stapleton D, Michell BJ, Chen ZP, Witters LA: Dealing with energy demand: the AMP-activated protein kinase. *Trends Biochem Sci* 24:22–25, 1999
15. Hardie DG, Carling D: The AMP-activated protein kinase: fuel gauge of the mammalian cell? *Eur J Biochem*. 246:259–273, 1997
16. Winder WW, Hardie DG: AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* 277:E1–E10, 1999
17. Ruderman NB, Saha AK, Vavvas D, Witters LA: Malonyl-CoA, fuel sensing, and insulin resistance. *Am J Physiol* 276:E1–E18, 1999
18. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ: Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47:1369–1373, 1998
19. Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, Goodyear LJ: Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49:527–531, 2000
20. Bergeron R, Russell RR 3rd, Young LH, Ren JM, Marcucci M, Lee A, Shulman GI: Effect of AMPK activation on muscle glucose metabolism in conscious rats. *Am J Physiol* 276:E938–E944, 1999
21. Russell RR III, Bergeron R, Shulman GI, Young LH: Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol* 277:H643–H649, 1999
22. Hardie DG, Carling D, Carlson M: The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* 67:821–855, 1998
23. Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, The T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE: Mammalian AMP-activated protein kinase subfamily. *J Biol Chem* 271:611–614, 1996
24. Thornton C, Snowden MA, Carling D: Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. *J Biol Chem* 273:12443–12450, 1998
25. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE: AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett* 443:285–289, 1999
26. Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR: A muscle-specific insulin receptor knockout challenges the current concepts of glucose disposal and NIDDM pathogenesis. *Mol Cell* 2:559–569, 1998
27. Lamprecht W, Stein P: Creatine phosphate. In *Methods of Enzymatic Analysis*. Bergmeyer H-U, Ed. New York, Academic, 1967, p. 610–611
28. Davies SP, Carling D, Hardie DG: Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur J Biochem* 186:123–128, 1989
29. Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR: Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol* 14:4902–4911, 1994
30. Clarke JF, Young PW, Yonezawa K, Kasuga M, Holman GD: Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. *Biochem J* 300:631–635, 1994
31. Goodyear LJ, Giorgino F, Balon TW, Condorelli G, Smith RJ: Effects of contractile activity on tyrosine phophoproteins and PI 3-kinase activity in rat skele-

tal muscle. *Am J Physiol* 268:E987–E995, 1995
32. Lee AD, Hansen PA, Holloszy JO: Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose transport activity in skeletal muscle. *FEBS Lett* 361:51–54, 1995
33. Yeh JI, Gulve EA, Rameh L, Birnbaum MJ: The effects of wortmannin on rat skeletal muscle: dissociation of signaling pathways for insulin- and contraction-activated hexose transport. *J Biol Chem* 270:2107–2111, 1995
34. Lund S, Holman GD, Schmitz O, Pedersen O: Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proc Natl Acad Sci U S A* 92:5817–5821, 1995
35. Kapur S, Bédard S, Marcotte B, Côté CH, Marette A: Expression of nitric oxide synthase in skeletal muscle: a novel role for nitric oxide as a modulator of insulin action. *Diabetes* 46:1691–1700, 1997
36. Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D: Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J* 17:1688–1699, 1998
37. Vavvas D, Apazidis A, Saha AK, Gamble J, Patel A, Kemp BE, Witters LA, Ruderman NB: Contraction-induced changes in acetyl-CoA carboxylase and 5′-AMP-activated kinase in skeletal muscle. *J Biol Chem* 272:13255–13261, 1997
38. Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Birnbaum MJ, Witters LA, Thorell A, Goodyear LJ: Exercise induces isoform-specific increase in 5′AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* 273:1150–1155, 2000
39. Bédard S, Marcotte B, Marette A: Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase. *Biochem J* 325:487–493, 1997