Nitric Oxide Stimulates Skeletal Muscle Glucose Transport Through a Calcium/Contraction- and Phosphatidylinositol-3-Kinase–Independent Pathway

Garret J. Etgen, Jr., David A. Fryburg, and E. Michael Gibbs

Recently published data have provided evidence that nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) are signaling intermediates in the pathway through which muscle contraction stimulates glucose transport. As exercise promotes both NO production and calcium flux, we examined the relationships between NO-stimulated glucose uptake and calcium-, contraction-, and phosphatidylinositol-3-kinase (PI-3-K)–mediated glucose transport in the isolated incubated rat epitrochlearis muscle preparation. The NO donor sodium nitroprusside (SNP; 10 mmol/l) and dibutyryl cGMP (100 μmol/l) accelerated epitrochlearis glucose transport four- to fivefold above basal levels ($P < 0.001$) in a manner similar to in vitro contractile activity and the calcium releasing agent *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7; 100 μmol/l). In the case of SNP, this effect could be completely attributed to an increase in cell surface GLUT4. The effect of SNP on glucose transport was not inhibitable by either wortmannin (1.5 μmol/l) or dantrolene (12.5 μmol/l). Similarly, neither calcium nor contraction stimulation of glucose transport was affected by the NO synthase inhibitors N^G-monomethyl-L-arginine (L-NMMA; 100 μmol/l) or 7-nitroindazole (1 mmol/l). Furthermore, whereas SNP raised epitrochlearis cGMP levels tenfold ($P < 0.001$), neither in vitro contractile activity nor W7 significantly elevated cGMP. These results indicate that NO/cGMP can markedly stimulate skeletal muscle glucose transport by increasing GLUT4 levels at the cell surface by a mechanism that does not depend on activation of PI-3-K. In addition, since calcium/contraction–stimulated glucose transport is not blocked by NO synthase inhibition and did not elevate cGMP, NO/cGMP may be part of a novel pathway that is distinct from both the insulin- and contraction-activated mechanisms. *Diabetes* 46:1915–1919, 1997

From Pfizer Central Research, Departments of Cardiovascular and Metabolic Diseases and Early Clinical Development, Groton, Connecticut.

Address correspondence and reprint requests to E. Michael Gibbs, PhD, Department of Cardiovascular and Metabolic Diseases, Pfizer Central Research, Groton, CT 06340. E-mail: gibbsem@pfizer.com.

Received for publication 22 July 1997 and accepted 13 August 1997.

BSA, bovine serum albumin; cGMP, cyclic guanosine monophosphate; eNOS, endothelial NO synthase; KHB, Krebs-Henseleit bicarbonate; L-NMMA, N^G-monomethyl-L-arginine; nNOS, neuronal NO synthase; PI-3-K, phosphatidylinositol-3-kinase; SNP, sodium nitroprusside; TCA, trichloroacetic acid; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

Insulin-stimulated postprandial glucose disposal occurs primarily in skeletal muscle. As a result, the transport of glucose across the plasma membrane, which is a rate-limiting step in the disposal process (1), is a key event in the maintenance of whole-body glucose homeostasis. Both insulin and muscle contraction accelerate glucose transport in skeletal muscle by increasing cell surface GLUT4 levels, although the biochemical mechanisms by which this occurs are distinct for the two stimuli (2–5). In contrast to our greater understanding of the pathway by which insulin stimulates glucose transport, relatively little is known about the biochemical pathway by which muscle contraction elicits this response. Currently, it is believed that contraction stimulates glucose transport by mobilizing an insulin-insensitive GLUT4 pool (6), appears to be directly related to calcium release (7,8), and does not use key insulin signaling intermediates such as phosphatidylinositol-3-kinase (PI-3-K) (9,10).

Balon and Nadler (11,12) recently suggested that exercise-stimulated glucose transport may be mediated by nitric oxide (NO). In those studies, in vivo motor nerve stimulation increased muscle NO release (11), and contraction-stimulated glucose transport was substantially reduced by inhibition of NO synthase (12). This inhibition was specific to the contractile pathway, as N^G-monomethyl-L-arginine (L-NMMA), a competitive NO synthase inhibitor, had no effect on insulin-stimulated glucose transport (12). On the basis of these findings and the fact that both endothelial and neuronal NO synthases (eNOS and nNOS, respectively) require calcium as a cofactor for activation (13), we hypothesized that during excitation-contraction coupling, calcium released from the sarcoplasmic reticulum stimulates the production of NO. NO in turn activates guanylyl cyclase, thereby increasing cyclic guanosine monophosphate (cGMP), which through an as yet unknown mechanism increases glucose transport. The purpose of the present investigation was to determine the relationships between NO-stimulated glucose transport and calcium-, contraction-, and PI-3-K–mediated glucose transport.

RESEARCH DESIGN AND METHODS

Materials. Sodium nitroprusside (SNP), wortmannin, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), dantrolene, fraction V bovine serum albumin (BSA), D-glucose, mannitol, sodium pyruvate, and 3-*O*-methyl-D-glucose (3-OMG) were obtained from Sigma Chemical (St. Louis, MO). 3-*O*-methyl-D[^{3}H]glucose and [1-^{14}C]mannitol were purchased from Du Pont New England

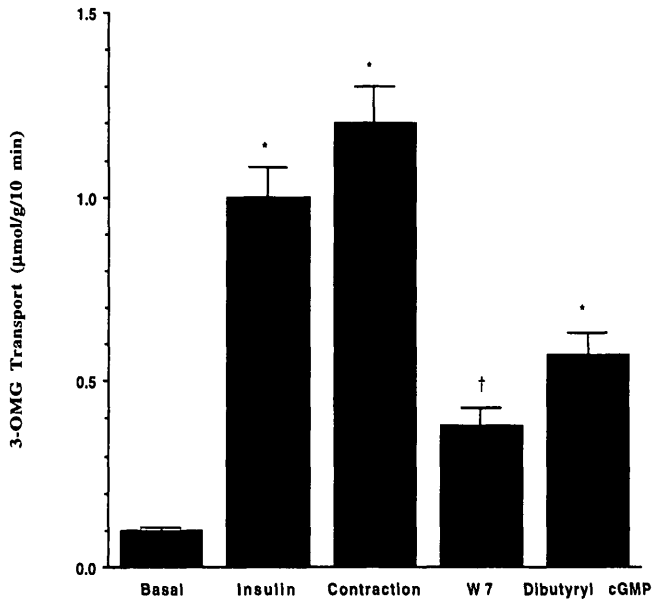

FIG. 1. Isolated rat epitrochlearis 3-OMG transport activity in the absence of stimulation, in the presence of 333 nmol/l insulin, 100 μmol/l W7, 100 μmol/l dibutyryl cGMP, or following in vitro muscle contractile activity: *significantly different than basal, $P < 0.001$; †significantly different than basal, $P < 0.01$.

Nuclear (Boston, MA). L-NMMA and guanosine 3′,5′-cyclic monophosphate, N^2,2′-*O*-dibutyryl-, sodium salt (dibutyryl cGMP) were purchased from Calbiochem (La Jolla, CA). The impermeant glucose transporter photoaffinity reagent 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-[2-^{3}H]BMPA) (specific activity 10 Ci/mmol) was kindly provided by G.D. Holman (University of Bath, U.K.).

Assessment of glucose transport activity. Nonfasted female Sprague-Dawley rats (Charles River, Boston, MA) weighing ~100 g were anesthetized via an intraperitoneal injection of pentobarbital sodium (6.5 mg/100 g body wt). Epitrochlearis muscles were then isolated and incubated individually in a manner similar to that described previously (5). Epitrochlearis muscles were used because their architectural nature (~12 fibers thick) makes them ideally suited for isolated incubated metabolic studies because diffusion limitations that may confound results in larger diameter muscles with tubular architecture are minimized (14). Briefly, muscles were preincubated for 90 min (except for the experiment shown in Fig. 2 that had 60-min preincubation) at 29°C in 1.8 ml of continuously gassed (95% O_2–5% CO_2) Krebs-Henseleit bicarbonate buffer (KHB) containing 0.1% BSA, 8 mmol/l glucose, and 32 mmol/l mannitol. After the preincubation phase, muscles were washed in KHB (1.8 ml at 29°C) containing 0.1% BSA and 40 mmol/l mannitol. Muscles were then transferred to fresh KHB, and glucose transport was measured over 10 min at 29°C in the presence of 8 mmol/l 3-OMG (250 μCi/mmol 3-*O*-methyl-D[^{3}H]glucose), 30 mmol/l mannitol (10 μCi/mmol [1-^{14}C]mannitol), and 2 mmol/l sodium pyruvate. After the final incubation phase, muscles were blotted on gauze and then clamp-frozen in liquid N_2-cooled tongs. Glucose transport activity, expressed in micromoles per gram per 10 min, was calculated from the intracellular ^{3}H-3-OMG accumulation using [U-^{14}C]mannitol as the extracellular marker. When the effects of insulin, SNP, dibutyryl cGMP, L-NMMA, and wortmannin were examined, these compounds were present during preincubation, wash, and incubation phases. In contrast, W7 was present only during the 90-min preincubation phase.

The effects of muscle contraction on glucose transport activity were investigated using a specially designed contraction apparatus that has been described previously (5). Briefly, after isolation each epitrochlearis muscle was pinned at the approximate resting length in a contraction apparatus that is designed such that two platinum electrodes reside on either side of the muscle. Each apparatus was then inserted into a 10- × 16-cm tube filled with 3 ml KHB containing the same constituents described above for the preincubation phase. The continually gassed and pinned muscles were preincubated at 29°C for 60 min and then changed to fresh buffer for the contraction period. Muscles were stimulated to contract for 2 × 10 min separated by a 1 min rest via 200-ms trains of 100 Hz using a model S48 stimulator (Astro-Med, Grass Instruments, Warwick, RI) attached to a Stimu-Splitter (MedLab Instruments, Loveland, CO). The trains were delivered 2/s at 10 volts. Preliminary experiments revealed this contraction protocol to produce a maximal effect on epitrochlearis glucose transport activity. At the end of the contraction period, the pinned muscles were incubated for 10 min at 29°C in 3 ml KHB as described above for the wash period. Muscles were then removed from the contraction apparatus and treated exactly as described above for measurement of glucose transport activity.

Assessment of cell surface GLUT4 protein. Epitrochlearis muscles were preincubated in the presence or absence of 10 mmol/l SNP and washed exactly as described above for the assessment of glucose transport. After the wash period, however, the muscles were transferred to 1 ml KHB containing 0.1% BSA, 1.5 μCi/ml ATB-[2-^{3}H]BMPA, and the presence or absence of 10 mmol/l SNP for 5 min. Muscles were then irradiated for 2 × 2.5 min in a Rayonet RPR 200 photochemical reactor (Southern New England Ultraviolet, Branford, CT). Muscles were turned over between irradiation intervals to achieve maximum exposure. Subsequent to irradiation, the muscles were trimmed and clamp-frozen in liquid N_2-cooled tongs.

Photolabeled muscles were processed for cell surface GLUT4 as described previously (15) except the gel cross-linking agent *N,N′*-diallyltartardiamide (DATD) was used instead of *N,N′*-methylenebisacrylamide, which greatly simplified the processing procedure. Labeled GLUT4 is expressed in disintegrations per minute/milligram wet weight.

Measurement of cGMP. Epitrochlearis muscles were incubated with the experimental perturbations as described above for measurement of glucose transport activity. However, after the preincubation phase in the absence or presence of test compound or after electrical stimulation, the muscles were rapidly clamp-frozen in liquid N_2-cooled tongs. Frozen muscles were homogenized on ice via polytron in 1 ml 10% trichloroacetic acid (TCA). Homogenates were centrifuged for 10 min at 14,000*g* and then the TCA was extracted from the supernatant with dH_2O saturated diethyl ether. Samples were then immediately frozen in liquid N_2 and concentrated in a speed vac. The concentrated samples were resuspended in assay buffer and acetylated, and then cGMP levels were measured by immunoassay according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). cGMP is expressed as picomoles per gram wet weight.

Statistical analysis. All data are expressed as means ± SE. The data were analyzed via analysis of variance with significant differences between means identified using Fisher's protected least significant difference (PLSD).

RESULTS

Stimulation of epitrochlearis glucose transport activity. As depicted in Fig. 1, insulin and electrically stimulated muscle contraction accelerate epitrochlearis glucose transport activity ~10- to 12-fold, respectively ($P < 0.001$). The calcium releasing agent W7 stimulated epitrochlearis glucose transport activity (~3.5-fold above basal; $P < 0.01$) in the

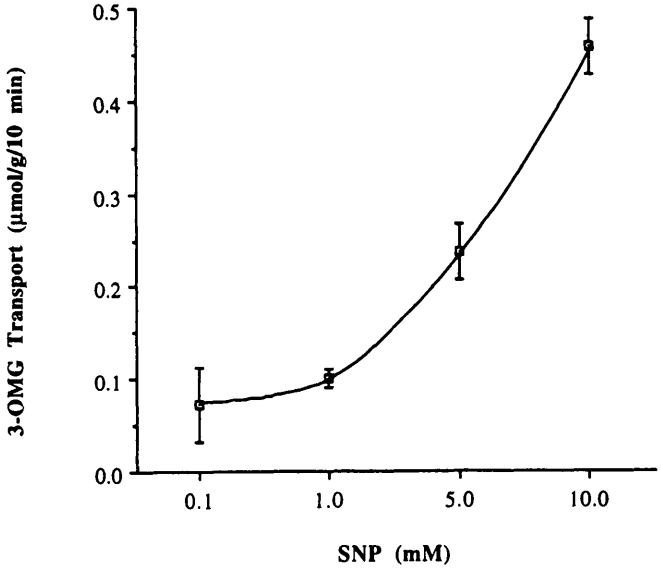

FIG. 2. Dose-response effect of SNP (60-min preincubation) on isolated rat epitrochlearis 3-OMG transport activity.

absence of any perceivable change in muscle length (Fig. 1) demonstrating that calcium stimulates muscle glucose transport when present at subcontracture levels. In addition, Fig. 1 shows that dibutyryl cGMP accelerated epitrochlearis glucose transport activity ~sixfold over basal levels ($P < 0.001$).

The NO donor, SNP, dose-dependently increased glucose transport in skeletal muscle up to ~fivefold over basal levels at the highest dose tested (10 mmol/l; Fig. 2). Furthermore, cell surface GLUT4 was increased fourfold by 10 mmol/l SNP (Fig. 3).

Dissociation of stimulatory pathways.

PI-3-K inhibition. The effect of the PI-3-K inhibitor, wortmannin, on insulin-, SNP-, and W7-stimulated epitrochlearis glucose transport activity is depicted in Fig. 4. Whereas wortmannin inhibited insulin-stimulated glucose transport by 95%, neither W7- nor SNP-stimulated glucose transport was affected;

NO synthase inhibition. L-NMMA at 10 μmol/l (not shown) and 100 μmol/l had no effect on basal, W7-, insulin-, or contraction-stimulated epitrochlearis glucose transport activity (Fig. 5). Similarly, another inhibitor of NO synthase, 7-nitroindazole (1 mmol/l), failed to inhibit W7-stimulated glucose transport activity (data not shown). These results suggested that neither subcontracture intracellular calcium release nor contraction stimulates glucose transport through the NO pathway.

Inhibition of calcium release. W7- and SNP-treated epitrochlearis muscles were coincubated with dantrolene, which inhibits calcium release from the sarcoplasmic reticulum. Dantrolene (12.5 μmol/l) completely abolished the effect of W7, but did not alter the ability of SNP to stimulate epitrochlearis glucose transport (Fig. 6).

cGMP elevation. SNP markedly increased epitrochlearis cGMP levels ($P < 0.001$), whereas insulin and W7 did not affect cGMP (Fig. 7). A small, statistically insignificant increase in cGMP was observed following contraction.

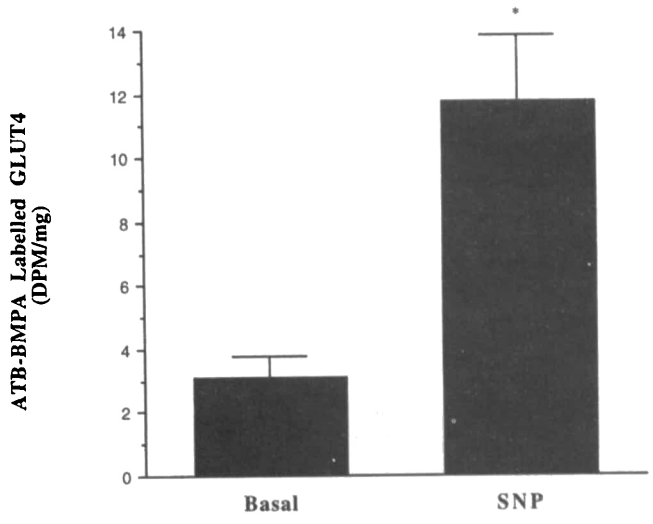

FIG. 3. Isolated rat epitrochlearis ATB-BMPA–labeled cell surface GLUT4 in the absence of stimulation and following 90-min exposure to 10 mmol/l SNP. *Significantly different from basal, $P < 0.001$.

DISCUSSION

Consistent with previous observations, this study demonstrated that SNP, W7, and electrically stimulated contraction all increase skeletal muscle glucose transport (4,7,12,16). This study extends the earlier observations by demonstrating the following: *1*) the ability of SNP to enhance glucose transport is due to GLUT4 translocation; and *2*) the increase in glucose transport due to SNP is neither calcium- nor PI-3-K–dependent. These results point to a possible novel role for NO in mediating muscle glucose utilization.

In the present study, SNP stimulated glucose transport in isolated epitrochlearis muscle, which is in agreement with very recent results obtained in isolated extensor digitorum longus (12) and soleus (16) muscles. The increase in glucose transport produced by SNP in the present study (fivefold, Figs. 2, 4, and 6) is somewhat greater than that observed in the two earlier studies, which was ~twofold. Although methodologi-

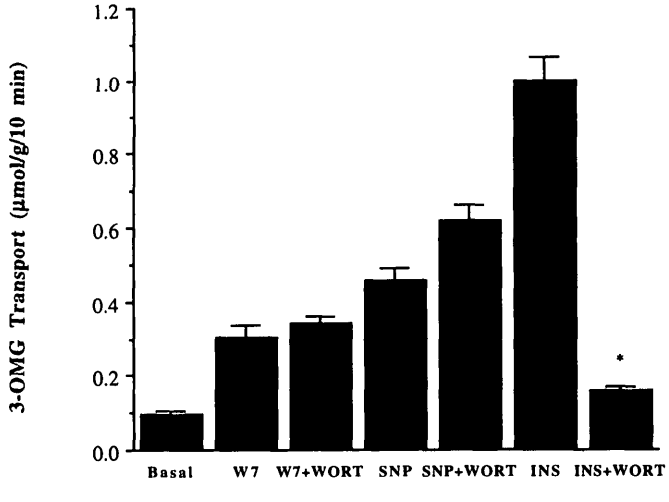

FIG. 4. Effect of 1.5 μmol/l wortmannin (WORT) on 100 μmol/l W7, 10 mmol/l SNP, or 333 nmol/l insulin (INS)-stimulated 3-OMG transport activity in isolated rat epitrochlearis muscle. *Significantly different from corresponding nonwortmannin treatment, $P < 0.001$.

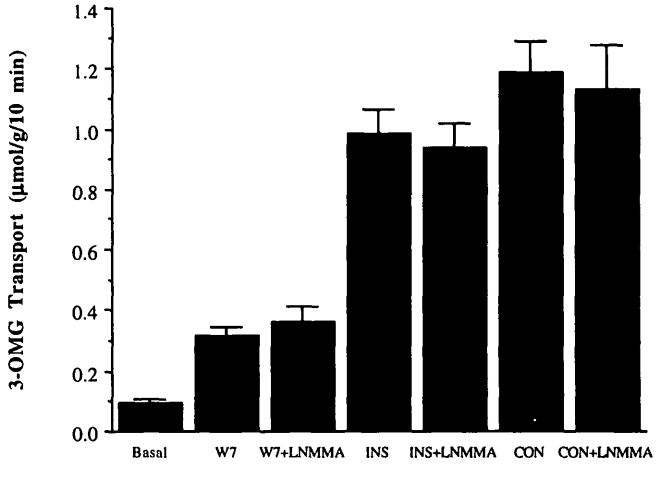

FIG. 5. Effect of 100 μmol/l L-NMMA on 100 μmol/l W7, 333 nmol/l insulin (INS), or contraction (CON)-stimulated 3-OMG transport activity in isolated rat epitrochlearis muscle.

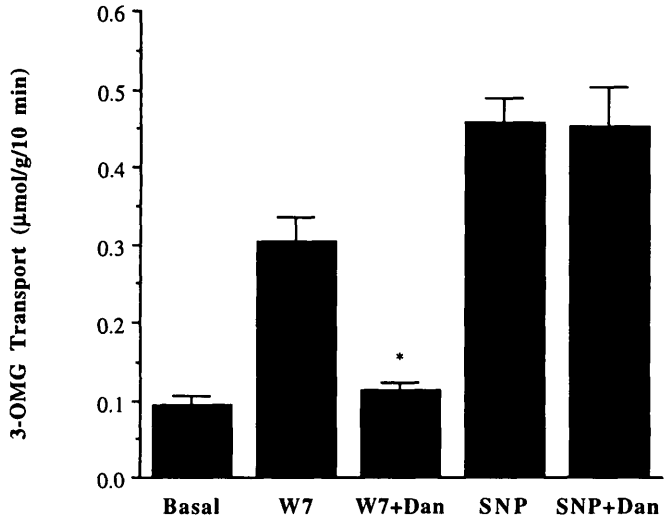

FIG. 6. Effect of 12.5 μmol/l dantrolene (Dan) on 100 μmol/l W7 or 10 mmol/l SNP–stimulated 3-OMG transport activity in isolated rat epitrochlearis muscle. *Significantly different from corresponding nondantrolene treatment, $P < 0.01$.

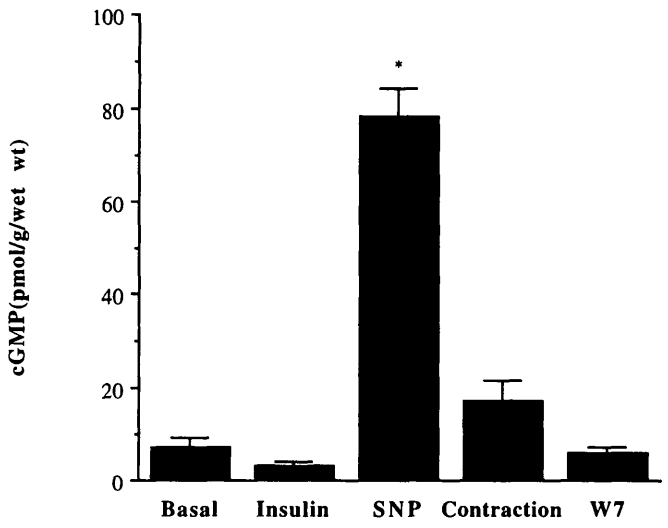

FIG. 7. Isolated rat epitrochlearis cGMP levels following 90-min incubations in the absence of stimulation, in the presence of 333 nmol/l insulin, in the presence of 100 μmol/l W7, or following in vitro muscle contractile activity. *Significantly different from basal, $P < 0.001$.

cal differences may account for part of the quantitative difference, the muscle type used may also contribute; nNOS expression differs between muscle fiber types, with the highest expression levels found in fast-twitch glycolytic muscles, such as the epitrochlearis, and the lowest in slow-twitch muscles, such as the soleus (17). Therefore, NO–signaling capacity may be greater in the epitrochlearis than the soleus, resulting in the observed differences in SNP stimulatory capacity. A systematic investigation of NO effects on carbohydrate metabolism in different muscle fiber types is needed.

To determine whether SNP, like insulin and muscle contraction, stimulates glucose transport by increasing GLUT4 protein at the cell surface, we employed the membrane impermeant photolabeling reagent, ATB-[2-^{3}H]BMPA, to quantitate cell surface GLUT4. In all likelihood, the SNP-induced increase in glucose transport is mediated via GLUT4 translocation to the cell surface since 10 mmol/l SNP was shown to increase cell surface GLUT4 to essentially the same magnitude as the increase in glucose transport (fourfold and fivefold, respectively; Figs. 3 and 2).

Previously, it has been demonstrated that the effect of calcium on glucose transport is not contraction dependent per se, but rather appears to be directly related to the level of intracellular calcium (7,8). In agreement with this observation, we found that the calcium releasing agent, W7, stimulated glucose transport activity in the absence of a perceivable change in muscle length. The specificity of the W7 action was verified by the use of dantrolene, which blocks calcium release from the sarcoplasmic reticulum and which completely abolished the stimulatory effect of W7. Dantrolene did not block SNP-stimulated glucose transport, which indicates that calcium release is not required for NO to mediate glucose transport stimulation. On the other hand, if calcium/contraction uses NO as a signaling intermediate, NO synthase inhibition should block, at least in part, the ability of calcium/contraction to stimulate muscle glucose transport.

Unlike the study of Balon and Nadler (12), L-NMMA in the present study did not inhibit contraction-stimulated or calcium-induced glucose transport. These results suggest that under the specific conditions examined, neither contraction nor calcium use NO as a signaling intermediate in the stimulation of glucose transport. It is important to note, however, that major differences in experimental protocols were used in the study by Balon and Nadler (12) and the current investigation. All of our experiments, including contraction of the muscle, used the isolated incubated epitrochlearis preparation, whereas Balon and Nadler (12) induced muscle contraction in vivo via electrical stimulation of the sciatic nerve and then isolated the extensor digitorum longus muscle for glucose transport assessment following appropriate drug incubation ex vivo. Because of these substantial experimental differences, it is likely that the two studies represent investigations of somewhat different phenomena and that the precise relationship between exercise and NO-mediated enhancement of glucose transport remains to be clarified.

Similarly, the lack of effect of wortmannin on SNP–induced glucose transport suggests that NO either is not involved in the major insulin-signaling pathway (PI-3-K) or lies downstream of this effector. Taken together, the results from this investigation indicate that NO is not a required signaling intermediate in the pathway by which insulin, subcontracture intracellular calcium, or contraction stimulate glucose transport.

Importantly, our results do not rule out an important role for NO/cGMP participation in the metabolic response to exercise. For instance, NO clearly increases muscle blood flow, which coincidentally enhances glucose delivery (18). However, in order for glucose extraction to be efficient at higher flow rates, the number of transporters at the cell surface must increase as well. NO fulfills this need as documented in the current study. Further, Young et al. (16) suggested that stimulation of the NO/cGMP system may be involved in the inactivation of glycogen synthase. Clearly, a redirection of muscle glucose disposal from the storage pathway to oxidation in times of increased energy requirement would be beneficial. Thus, it may be suggested that during repeated in vivo muscle contraction, or exercise, the modu-

latory role of NO to exert the proper metabolic adjustments may be of critical importance for sustaining the activity.

In summary, results from the present study demonstrated the following:

1) NO stimulates skeletal muscle glucose transport by increasing cell surface GLUT4 protein;
2) the effect on glucose transport is mimicked by a long-acting cGMP analog;
3) the NO/cGMP effect is not PI-3-K– or calcium-dependent; and
4) insulin, calcium, and electrically stimulated contraction do not exert their stimulatory effects on glucose transport via the NO pathway.

We suggest that NO/cGMP represents a novel, insulin- and contraction-independent, pathway for stimulating muscle glucose transport.

ACKNOWLEDGMENTS

We thank Thomas W. Balon (Duarte, CA) for providing us with reprints of some of his work prior to its publication.

REFERENCES

1. Ren JM, Marshall BA, Gulve EA, Gao J, Johnson DW, Holloszy JO, Mueckler M: Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem* 268: 16113–16115, 1993
2. Lund S, Holman GD, Schmitz O, Pedersen O: GLUT4 content in the plasma membrane of rat skeletal muscle: comparative studies of the subcellular fractionation method and the exofacial photolabelling technique using ATB-BMPA. *FEBS Lett* 330:312–318, 1993
3. Wilson CM, Cushman SW: Insulin stimulation of glucose transport activity in rat skeletal muscle: increase in cell surface GLUT4 as assessed by photolabelling. *Biochem J* 299: 755–759, 1984
4. 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 USA* 92: 5817–5821, 1995
5. Etgen GJ Jr, Wilson CM, Jensen J, Cushman SW, Ivy JL: Glucose transport and cell surface GLUT-4 protein in skeletal muscle of the obese Zucker rat. *Am J Physiol* 271: E294–E301, 1996
6. Coderre L, Kandror KV, Vallega G, Pilch PF: Identification and characterization of an exercise-sensitive pool of glucose transporters in skeletal muscle. *J Biol Chem* 270: 27584–27588, 1995
7. Youn JH, Gulve EA, Holloszy JO: Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction. *Am J Physiol* 260: C555–C561, 1991
8. Youn JH, Gulve EA, Henriksen EJ, Holloszy JO: Interactions between effects of W-7, insulin, and hypoxia on glucose transport in skeletal muscle. *Am J Physiol* 267: R888–R894, 1994
9. 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
10. Yeh JI, Gulve EA, Rameh L, Birnbaum MJ: The effects of wortmannin on rat skeletal muscle. *J Biol Chem* 270: 2107–2111, 1995
11. Balon TW, Nadler JL: Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol* 77:2519–2521, 1994
12. Balon TW, Nadler JL: Evidence that nitric oxide increases glucose transport in skeletal muscle. *J Appl Physiol* 82:359–363, 1997
13. Schmidt HHHW, Lohman SM, Walter U: The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim Biophys Acta* 1178: 153–175, 1993
14. Bonen A, Clark MG, Henriksen EJ: Experimental approaches in muscle metabolism: hindlimb perfusion and isolated muscle incubations. *Am J Physiol* 266: E1–E16, 1994
15. Etgen GJ Jr, Jensen J, Wilson CM, Hunt DG, Cushman SW, Ivy JL. Exercise training reverses insulin resistance in muscle by enhancing recruitment of GLUT4 to the cell surface. *Am J Physiol* 272: E864–E869, 1997
16. 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
17. Kobzik L, Reid MB, Bredt DS, Stamler JS: Nitric oxide in skeletal muscle. *Nature Lond* 372: 546–548, 1994
18. Segal SS: Cell-to-cell communication coordinates blood flow control. *Hypertension Dallas* 23: 1113–1120, 1994