Nitric oxide increases GLUT4 expression and regulates AMPK signaling in skeletal muscle

Vitor A. Lira, Quinlyn A. Soltow, Jodi H. D. Long, Jenna L. Betters, Jeff E. Sellman, and David S. Criswell

Department of Applied Physiology and Kinesiology, Center for Exercise Science, University of Florida, Gainesville, Florida

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Lira VA, Soltow QA, Long JH, Betters JL, Sellman JE, Criswell DS. Nitric oxide increases GLUT4 expression and regulates AMPK signaling in skeletal muscle. Am J Physiol Endocrinol Metab 293: E1062–E1068, 2007. First published July 31, 2007; doi:10.1152/ajpendo.00045.2007.—Nitric oxide (NO) and 5'-AMP-activated protein kinase (AMPK) are involved in glucose transport and mitochondrial biogenesis in skeletal muscle. Here, we examined whether NO regulates the expression of the major glucose transporter in muscle (GLUT4) and whether it influences AMPK-induced upregulation of GLUT4. At low levels, the NO donor S-nitroso-N-penicillamine (SNAP, 1 and 10 μM) significantly increased GLUT4 mRNA (~3-fold; P < 0.05) in L6 myotubes, and cotreatment with the AMPK inhibitor compound C ablated this effect. The cGMP analog 8-bromo-cGMP (8-Br-cGMP, 2 mM) increased GLUT4 mRNA by ~50% (P < 0.05). GLUT4 protein expression was elevated 40% by 2 days treatment with 8-Br-cGMP, whereas 6 days treatment with 10 μM SNAP increased GLUT4 expression by 65%. Cotreatment of cultures with the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one prevented the SNAP-induced increase in GLUT4 protein. SNAP (10 μM) also induced significant phosphorylation of α-AMPK and acetyl-CoA carboxylase and translocation of phosphorylated α-AMPK to the nucleus. Furthermore, L6 myotubes exposed to 5-aminomidazole-4-carboxamidine-1β-ribofuranoside (AICAR) for 16 h presented an approximately ninefold increase in GLUT4 mRNA, whereas cotreatment with the non-isoinform-specific NOS inhibitor Nω-nitro-l-arginine methyl ester, prevented ~70% of this effect. In vivo, GLUT4 mRNA was increased 1.8-fold in the rat plantaris muscle 12 h after AICAR injection, and this induction was reduced by ~50% in animals cotreated with the neuronal and inducible nitric oxide synthases selective inhibitor 1-(2-trifluoromethylphenyl)-imidazole. We conclude that, in skeletal muscle, NO increases GLUT4 expression via a cGMP- and AMPK-dependent mechanism. The data are consistent with a role for NO in the regulation of AMPK, possibly via control of cellular activity of AMPK kinases and/or AMPK phosphatases.

5-aminimidazole-4-carboxamidine-1β-ribofuranoside; guanosine 3',5'-cyclic monophosphate; L6 myotubes; compound C; Nω-nitro-l-arginine methyl ester; glucose transporter 4; adenosine 5'-monophosphate-activated protein kinase

Skeletal muscle accounts for 65–90% of the clearance of an oral or intravenous glucose challenge (9, 24). Furthermore, muscle contractile activity augments glucose clearance by improving insulin sensitivity in normal and insulin-resistant rats and humans (10, 22, 25) and upregulating expression of the glucose transporter GLUT4 (15, 29). Previous studies have focused on the role of pathophysiologic levels of nitric oxide (NO) as a negative modulator of GLUT4 expression (3, 34). However, it remains elusive whether physiological concentrations of NO positively affect GLUT4 levels.

NO is a reactive nitrogen molecule that is formed enzymatically by nitric oxide synthase (NOS), via the conversion of l-arginine to l-citrulline. Skeletal muscle expresses neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) NOS isoforms (35, 36). eNOS and nNOS synthesize NO at lower levels, whereas iNOS expression increases during inflammation and acute exercise (23) and induces much higher NO production (35). NO synthesis increases during skeletal muscle contraction (2, 33, 36), and many of its signaling effects are mediated through activation of soluble guanylate cyclase (sGC), leading to increased production of cGMP (26, 35).

Both NO and cGMP are involved in mitochondrial biogenesis in different cell types (30, 31). Skeletal muscle GLUT4 upregulation seems to share several similarities with mitochondrial biogenesis, such as 5'-AMP-activated protein kinase (AMPK) participation and transcription stimulation by myocyte enhancer factor 2, as well as peroxisome proliferator-activated receptor-γ coactivator 1α (28, 31, 43, 44). At present, it is unknown whether NO integrates the AMPK-dependent pathway for GLUT4 upregulation.

The enzyme AMPK is heterotrimetric (α1, α2, β1, β2, γ1, γ2, γ3), and is sensitive to increases in the AMP-to-ATP ratio, being activated by both direct AMP allosteric regulation and phosphorylation, which is mediated by an upstream AMPK kinase (17, 41). Acutely, AMPK influences glucose transport and fatty acid oxidation in skeletal muscle (1, 37) while delayed responses related to AMPK activation include increased expression of GLUT4, mitochondrial proteins, and several metabolic enzymes in rat and human skeletal muscle. These changes represent important adaptive responses triggered by metabolic challenges such as exercise, energy deprivation, and hypoxia (39, 42, 43, 44).

The interaction between AMPK, NOS enzymes, and NO levels in muscle is intriguing. AMPK activation is known to inhibit iNOS expression (34). Conversely, AMPK is capable of phosphorylating and activating both eNOS and nNOS (5–7). Furthermore, the AMP analog 5-aminomidazole-4-carboxamidine-1β-ribofuranoside (AICAR) increases NO activity in H-2Kb muscle cells (12). Interestingly, high concentrations of the NO donor sodium nitroprusside (SNP, 5 and 10 mM) were found to increase phosphorylation of α1-AMPK in isolated rat extensor digitorum longus muscles (19). Altogether, these observations point toward the existence of a positive feedback interaction between AMPK and NOS in skeletal muscle.

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In this study, we tested the following two main hypotheses in skeletal muscle: 1) NO and cGMP signaling induce upregulation of GLUT4 expression, and 2) NO synthase (NOS) activity is required for the AMPK-induced increase in GLUT4 expression. Our findings show that both NO and cGMP increase GLUT4 mRNA and protein expression in L6 myotubes. Furthermore, we show that AICAR-induced upregulation in GLUT4 mRNA is repressed by NOS inhibition in cultured myotubes and in the plantaris muscle in vivo. Finally, we report that an NO donor activates AMPK and that inhibition of NOS activity prevents AICAR-induced activation of AMPK. Based on these data, a model of NO regulation of AMPK activity is proposed.

MATERIALS AND METHODS

Chemicals. AICAR was obtained from Toronto Research Chemicals (North York, ON). N\textsuperscript{3}-nitro-L-arginine methyl ester (L-NAME), 1-(2-trifluoromethyl-phenyl)-imidazole (TRIM), 5-nitroso-N-penicillamine (SNAP) and 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ) were purchased from Cayman Chemical (Ann Arbor, MI). 8-Bromo-cGMP (8-Br-cGMP) was obtained from Tocris Bioscience (Ellisville, MO), and compound C was from Calbiochem (San Diego, CA).

Animals. The University of Florida Institutional Animal Care and Use Committee approved the protocol of this study. Female Sprague-Dawley rats (10 mo old, ~300 g) were purchased from Harlan (Indianapolis, IN). Rats were injected intraperitoneally with AICAR (0.5 g/kg body wt) and/or TRIM (50 mg/kg body wt). Control animals were injected with saline in a volume proportional to the AICAR-treated animals (0.1 ml/10 g body wt). Control animals were injected with saline in a volume proportional to the AICAR-treated animals (0.1 ml/10 g body wt). TRIM was injected 1 h before AICAR to ensure that nNOS activity would already be reduced when the muscle was exposed to AICAR. The plantaris muscle was removed 12 h postinjections (21). Blood lactate assessed at the time of death was not affected by AICAR treatment (data not shown). Total RNA was isolated from muscle tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Muscle samples were also harvested from separate animals 1 h postinjection and homogenized in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM glycyr rhizate phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM PMSF, and 10 μg/ml aproatin containing 1% vol/vol phosphate inhibitor (p-5726) from Sigma.

Cell culture. Rat L6 myoblasts were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C in 5% CO₂ and 95% atmospheric air. Myoblasts were initially maintained in growth medium (10% FBS) containing 5 mM glucose DMEM supplemented with 1 mM L-carnitine, 10 mM creatine, 0.5 mM oleic acid, 100 U/ml penicillin, and 100 μg/ml streptomycin until reaching 80% confluence. Differentiation was induced by switching to medium containing 2% horse serum (HoS) for at least 7 days before treatments were begun. Treatment medium contained 10% HoS, 5% FBS (32), and one or more of the following chemicals: AICAR, compound C, SNAP, 8-Br-cGMP, 1-NAME, TRIM, or ODQ. Microscopic inspection of cells and removed media verified that cultures remained confluent myotubes throughout the treatment period and that none of the treatments negatively affected cell survival.

Cells were treated for 16 h in experiments examining the effects of SNAP and 8-Br-cGMP on GLUT4 mRNA levels, washed one time in ice-cold PBS containing 1 μM Na₂VO₄, and immediately harvested in TRIZol Reagent (Invitrogen). In the experiment looking at the effect of NOS inhibition on AMPK signaling, cells were treated for 16 h, washed two times with warm PBS, left in medium without treatments for 5 h, and then harvested as described above. To test the effects of cGMP and NO on GLUT4 protein expression, cells were treated for either 2 or 6 days with fresh medium, with treatments being added every day. To test the effects of NO levels and NOS activity on AMPK phosphorylation, acetyl-CoA carboxylase (ACC) phosphorylation, and phosphorylated (p)-α-AMPK migration to the nucleus, treatments were performed for either 1 or 2 h, and cells were immediately harvested. Whenever treatments were used in combination, inhibitors of NOS (1-NAME and TRIM), αGC (ODQ), and AMPK (compound C) were added 30 min before other treatments. For total protein extracts, cells were washed two times in ice-cold PBS containing 1 μM Na₂VO₄, 0.05% vol/vol protease inhibitors (p-8340), and 0.5% vol/vol phosphatase inhibitors (p-5726) from Sigma (St. Louis, MO) and harvested in nonedentating lysis buffer containing 1% vol/vol Triton X-100, 0.3 M NaCl, 0.05 M Tris base, 5 mM EDTA, 3.1 μM Na₃ATP, 95 mM NaF, 22 μM Na₂VO₄, 0.1% vol/vol protease inhibitors, and 1% vol/vol phosphate inhibitors. For isolation of nuclear proteins, cells were harvested in ice-cold PBS containing 1 μM Na₂VO₄, 0.05% vol/vol protease inhibitors, and 0.5% vol/vol phosphate inhibitors, centriuged, and the resulting pellets were treated with NE-PER nuclear and cytosolic extraction reagents (Pierce Biotechnology, Rockford, IL) containing 0.1% vol/vol protease inhibitors and 1% vol/vol phosphate inhibitors.

Quantitative real-time PCR. Concentration and purity of the extracted RNA were measured spectrophotometrically (240, 260 and 280 nm absorbance in 1X Tris-EDTA buffer (Promega, Madison, WI). RT was performed using the SuperScript III First-Strand Synthesis System for RT-PCR according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA). Reactions were carried out using 1–2 μg of total RNA and 2.5 μM oligo(dT)₂₀ primers. First-strand cDNA was created with two units of RNase H and stored at −80°C.

Primers and probes for GLUT4 (GenBank NM_012751.1, assay no Rn00562597_m1) were obtained from the ABI Assays-on-Demand service and consisted of Taqman 5'-labeled FAM reporters and 3'-nonfluorescent quenchers. Primer and probe sequences from this service are proprietary and therefore are not reported. Primer and probe sequences also consisting of Taqman 5'-labeled FAM reporters and 3'-nonfluorescent quenchers for hypoxanthine-guanine phosphoribosyl transferase (HPRT) obtained from Applied Biosystems (Assays-by-Design) are: forward, 5'-GTGTT-GATACAGGCGACATGTGG-3'; reverse, 5'-AGTCAAGGGCATGAACTTC-3'. Quantitative real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA). Each 25-μl PCR reaction contained 1 μl of cDNA. In this technique, amplification of the fluorescently labeled probe sequence located between the PCR primers was monitored in real time during the PCR program. The number of PCR cycles required to reach a predetermined threshold of fluorescence (Cₜ) was determined for each sample. Samples were quantified relative to the Cₜ for a normalizing gene, HPRT, determined separately in the same sample. HPRT was used as the normalizer because its expression was not altered by treatments and its amplification efficiency was approximately equal to the GLUT4 gene. This procedure is referred to as the comparative Cₜ method (4).

Immunoblots. Protein concentrations were measured using the DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell lysates (16–30 μg) were run in 7.5, 10, or 12% SDS-PAGE gels for p-ACC, GLUT4, and both phospho- and total α-AMPK blots, respectively. Nuclear extracts (11 μg) were run in 12% SDS-PAGE gels for phospho-α-AMPK blots. The primary antibodies used were as follows: goat anti-GLUT4 (1:1,200 dilution; Santa Cruz), rabbit anti-(α)-AMPK and anti-phospho-(α)-AMPK (1:1,000 dilution; Cell Signaling), and rabbit anti-pACC (1:500 dilution; Upstate). Ponceau stain was used to control for loading. Reactions were developed by using the enhanced chemiluminescence detection reagents (ECL Plus; Amersham Biosciences, Buckinghamshire, UK), and protein levels were determined by densitometry (Kodak 1D Image Analysis Software version 3.6).

Nitrate plus nitrite measurements in serum and medium. Nitrate plus nitrite (NO₃⁻ + NO₂⁻) levels were measured in frozen culture media collected after 24-h incubations (27, 34). Samples were thawed, diluted 1:1 with PBS, and filtered with Millipore UltrafreeMC micro-
centrifuge filter units (10,000 mol wt cutoff). Aliquots from each sample were then analyzed in triplicate by using a fluorometric assay kit for NO$_3^-$/NO$_2^-$ (Cayman Chemical).

### Statistical analysis

Results were treated with either one-way ANOVA followed by the Fisher least-significant difference test or with an independent Student’s *t*-test when applicable. Statistical significance was set a priori at *P* < 0.05.

## RESULTS

**NO and cGMP increase GLUT4 mRNA and protein expression in skeletal muscle cells.** At the lowest concentrations studied (1 and 10 μM), the NO donor SNAP increased GLUT4 mRNA by approximately threefold. At higher concentrations, SNAP did not alter GLUT4 mRNA (100 – 300 μM), although the highest dose (1,000 μM) decreased it compared with control (Fig. 1). The AMPK inhibitor compound C (45 μM) tended to lower GLUT4 mRNA expression in myotubes (*P* < 0.06), and cotreatment with 10 μM SNAP and compound C (45 μM) completely ablated the SNAP-induced increase in GLUT4 transcripts. SNAP (10 μM) induced an ~2.5-fold increase in NO$_3^-$ + NO$_2^-$ levels in the culture medium [control = 4.28 ± 0.55 (SE) μM, SNAP (10 μM) = 10.55 ± 0.56 (SE) μM, *P* = 0.004]; however, we failed to detect a significant change in NO$_3^-$ + NO$_2^-$ levels with 1 μM SNAP treatment (4.81 ± 0.28 μM). In separate experiments, the cGMP analog 8-Br-cGMP (2mM) induced a 1.5-fold increase in GLUT4 mRNA (Fig. 2).

GLUT4 protein levels were elevated after a 2-day treatment with 8-Br-cGMP (Fig. 3A). GLUT4 protein expression following 2-day treatment with 10 μM SNAP tended to be increased, but did not reach statistical significance (data not shown). Therefore, separate myotube cultures were treated with 10 μM SNAP for 6 days, resulting in an ~65% increase in GLUT4 protein. Cotreatment with the guanylyl cyclase inhibitor ODQ (1 μM) prevented this effect (Fig. 3B).

**NOS inhibition prevents induction of GLUT4 mRNA by AICAR.** GLUT4 mRNA in L6 myotubes was increased by approximately ninefold 5 h after a 16-h incubation with 10 μM AICAR. GLUT4 mRNA induction by AICAR was prevented by the NOS inhibitor L-NIO (500 μM, Fig. 4).

### Fig. 1. Nitric oxide (NO)-induced upregulation of GLUT4 mRNA is 5'-AMP-activated protein kinase (AMPK) dependent. L6 myotubes were treated for 16 h with different concentrations of the NO donor S-nitroso-N-penicillamine (SNAP), compound C (CC, 45 μM), and SNAP (10 μM) + compound C (45 μM, S10 + CC) and harvested immediately afterward. GLUT4 mRNA was measured with real-time PCR and normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA. Results are expressed relative to control as means ± SE for 3–6 individual experiments. *P < 0.01 compared with control (*) and compared with SNAP (100 – 1,000 μM), compound C, and SNAP + compound C (#).

### Fig. 2. cGMP induces upregulation of GLUT4 mRNA. L6 myotubes were treated for 16 h with different concentrations of the cGMP analog 8-bromo-cGMP (8-Br-cGMP) and harvested immediately afterward. GLUT4 mRNA was measured with real-time PCR and normalized to HPRT mRNA. Results are expressed relative to control as means ± SE for 4–6 individual experiments. *P < 0.05 compared with control and 500 μM.

### Fig. 3. NO-induced upregulation of GLUT4 protein is cGMP dependent. A: L6 myotubes were treated for 2 days with or without the cGMP analog 8-Br-cGMP (2 mM). B: L6 myotubes were treated for 6 days with or without the NO donor SNAP (1 and 10 μM) and the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM). Results are expressed relative to control as means ± SE for 4–5 individual experiments. *P < 0.05 compared with control.
AICAR. However, cotreatment with the nonisoform-specific NOS inhibitor L-NAME prevented 70% of the mean AICAR-induced effect on GLUT4 mRNA and did not differ significantly from the control group (Fig. 4A).

Injection of AICAR in rats in vivo resulted in an ~80% increase in GLUT4 mRNA in the plantaris muscle 12 h postinjection that was blunted by cotreatment with the nNOS- and iNOS-specific inhibitor TRIM (Fig. 4B).

**Inhibition of NOS activity ablates AMPK activation by AICAR.** Treatment of L6 myotubes with varying doses of the AMPK-activating drug AICAR (1–4 mM) showed significant induction of AMPK phosphorylation at concentrations ≥2 mM (Fig. 5A). As previously shown (13), treatment with compound C did not affect basal levels of AMPK and ACC phosphorylation. Cotreatment with AICAR and compound C, L-NAME, or TRIM prevented the AICAR-induced increase in AMPK phosphorylation (Fig. 5, B and C) and ACC phosphorylation (Fig. 5, B and D).

**NO is sufficient to activate AMPK in L6 myotubes.** SNAP (10 μM) treatment caused a transient increase in AMPK phosphorylation (Fig. 5, B and C) and ACC phosphorylation (Fig. 5, B and D).

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**Fig. 4. Nitric oxide synthase (NOS) inhibition prevents 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)-induced upregulation of GLUT4 mRNA in skeletal muscle.** A: L6 myotubes (5–6 individual experiments) were treated for 16 h with or without AICAR (1 mM, A) and Nω-nitro-L-arginine methyl ester [l-NAME (LN), 100 μM] and harvested 5 h afterward. B: rats (4 animals/group) were injected with saline [controls (CTR)], AICAR (A), and/or 1-(2-trifluoromethyl-phenyl)-imidazole [TRIM (TR)] 12 h before death, and the plantaris muscle was frozen immediately (refer to materials and methods for details). Inset refers to a representative blot of samples harvested 1 h after injections. On both figures GLUT4 mRNA was measured with real-time PCR and normalized to HPRT mRNA. Results are expressed relative to control as means ± SE. *P < 0.02 compared with all other conditions.

**Fig. 5. AICAR-induced AMPK activation is prevented by NOS inhibition.** A: AICAR induced α-AMPK phosphorylation in L6 myotubes treated for 1 h. Underlined values were significantly different from control (P < 0.02). B: representative blots of phosphorylated α-AMPK, total α-AMPK, and phosphorylated acetyl-CoA carboxylase (ACC) in myotubes treated for 1 h with AICAR (3 mM) alone, or in combination with compound C (45 μM), L-NAME (100 μM), or TRIM (100 μM). C: phosphorylated-to-total α-AMPK ratio in response to the same treatments as in B. D: phosphorylated ACC expression in the same conditions as in B and C. All results are expressed relative to control as means ± SE for 4–6 individual experiments. *P < 0.05 compared with all other conditions.
phosphorylation (Fig. 6A), pACC (Fig. 6B), and nuclear phospho-AMPK (Fig. 6C) at 1 h that had returned to control levels at 2 h. Total AMPK was increased in the nuclear fraction at 1 h to the same extent as phospho-AMPK, causing the nuclear phospho-to-total AMPK ratio to be unchanged (data not shown).

DISCUSSION

In this study, we present the novel finding that the NO- and cGMP-dependent pathway upregulates GLUT4 mRNA and protein expression in skeletal muscle. Our observations also suggest that NOS activity is required for the AMPK-induced upregulation of GLUT4 mRNA both in vitro and in vivo. In addition, we provide evidence for a positive feedback interaction between NOS and AMPK enzymes in muscle. Although it was previously shown that the NO donor SNP induced phosphorylation and activation of α1-AMPK in muscle (19), to our knowledge, ours is the first study to show that low NO levels can increase α-AMPK phosphorylation, ACC phosphorylation, and AMPK translocation to the nucleus.

NO can bind to and inhibit cytochrome synthase activity (8) and creatine kinase activity (16), thereby providing a potential mechanism for NO to increase the AMP-to-ATP ratio within a cell and activate AMPK. However, the AMP mimetic drug AICAR bypasses the cellular energy signal by directly activating the AMPK enzyme. Therefore, the necessity of NOS activity for AICAR-induced GLUT4 expression (Fig. 4) and AMPK activation (Fig. 5) argues that, although NO may affect cellular energy status, a more direct influence on the AMPK pathway is also present.

Our finding that the NO donor SNAP is able to induce GLUT4 expression (Figs. 1 and 3) and AMPK activation (Fig. 6) suggests that NOS may act on GLUT4 regulation upstream...
of AMPK. Paradoxically, our finding that the NOS inhibitors t-NAME and TRIM prevent AICAR-induced AMPK activation (Fig. 5) and GLUT4 mRNA expression (Fig. 4) imply that NOS activity is required downstream of AMPK. Activity of the AMPK enzyme complex is regulated by phosphorylation of the α-subunit at 172Thr. This activation is accomplished by the activity of upstream NOS kinases, such as LKB1, as well as allosteric activation via AMP binding (17, 18, 42). Based on our data, we propose that NO may be involved in the regulation of AMPK kinase activity and/or inhibition of protein phosphatases responsible for AMPK dephosphorylation. Figure 7 shows this proposed model of AMPK regulation. Because much of the excitatory effects of AMP on AMPK activity are because of facilitation of phosphorylation (17), this model would explain why NOS inhibition interferes with activation of AMPK signaling by the AMP mimetic drug AICAR. The model would also be consistent with activation of AMPK by an NO donor, independent of cellular metabolic changes. Because α2-AMPK is the predominant isoform in skeletal muscle, and the one responsible for AICAR-induced glucose transport (13), it is likely that our results reflect a regulatory role of NO on α2-AMPK activation. Further studies should examine the potential role of NO and cGMP in the regulation of known AMPK kinases.

Different rates of NO production cause opposite adaptations in muscle. As previously suggested, the distinct effects of NO may be classified as cGMP dependent and cGMP independent (40). NO-mediated mitochondrial biogenesis and vasodilation are examples of cGMP-dependent effects, which are related to eNOS and nNOS activity, involving relatively low NO concentrations. On the other hand, cGMP-independent effects usually result from high NO levels and include nitrogen species-mediated nitrosative modification of proteins, lipids, and/or DNA (38). As an example, a 24-h incubation of L6 myotubes with cytokines and LPS induces iNOS expression, increases NO production (40- to 50-fold higher than controls), and decreases GLUT4 levels (3, 34). These studies provide evidence suggesting a role for iNOS expression and pathophysiological NO levels in insulin resistance. Our findings are consistent with these studies, since the highest concentration of SNAP (1,000 µM) decreased GLUT4 mRNA expression (Fig. 1).

NO-sensitive guanylyl cyclase responds to nanomolar concentrations of NO, with maximal activation reported in the range of 1–100 nM (11). Hirota et al. (20) reported NO concentration in culture media of 285 nM with 100 µM SNAP treatment. Therefore, our 1- and 10-µM treatments likely activated cGMP production. Therefore, we sought to test whether cGMP production was required for the observed effects on GLUT4 expression. Our results clearly show that the cGMP analog 8-Br-cGMP induced increases in GLUT4 mRNA (Fig. 2) and protein (Fig. 3A) expression. Furthermore, guanylyl cyclase inhibition with ODQ prevented the SNAP-induced increase in GLUT4 protein levels (Fig. 3B).

Our results in vivo also support our proposed model, since AICAR induced AMPK phosphorylation and increased GLUT4 mRNA levels in the plantaris muscle (Fig. 4B). Pilon et al. (34) provided evidence that AICAR inhibits iNOS expression. Thus, the fact that co-treatment of animals with the nNOS- and iNOS-specific inhibitor TRIM blunted the AICAR-induced increase in GLUT4 mRNA suggests that skeletal muscle nNOS is the major isoform involved in AMPK activation of GLUT4 expression in skeletal muscle.

The present study adds important information regarding the NO-cGMP pathway and the plasticity of skeletal muscle. More specifically, our experiments demonstrate that GLUT4 expression in L6 myotubes, both at mRNA and protein levels, is positively regulated by NO and cGMP. In addition, our results show that NOS activity is necessary for AICAR-induced activation of AMPK and expression of GLUT4. Our findings are consistent with a role for NO in the regulation of AMPK kinase activity, or perhaps inhibition of AMPK phosphatase activity. Understanding the mechanisms involved in GLUT4 regulation in skeletal muscle will aid in the development of effective treatments for metabolic disorders.

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