Role of the AMPKγ3 isoform in hypoxia-stimulated glucose transport in glycolytic skeletal muscle

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Deshmukh AS, Glund S, Tom RZ, Zierath JR. Role of the AMPKγ3 isoform in hypoxia-stimulated glucose transport in glycolytic skeletal muscle. Am J Physiol Endocrinol Metab 297: E1388–E1394, 2009. First published October 13, 2009; doi:10.1152/ajpendo.00125.2009.—Skeletal muscle glucose transport is regulated via the canonical insulin-signaling cascade as well as by energy-sensing signals. 5′-AMP-activated protein kinase (AMPK) has been implicated in the energy status regulation of glucose transport. We determined the role of the AMPKγ3 isoform in hypoxia-mediated energy status signaling and glucose transport in fast-twitch skeletal muscle. KN-62 and KN-93 have been used to determine the role of competitive inhibitors of CaM-dependent protein kinase (CaMK) on hypoxia-stimulated glucose transport in rat skeletal muscle. KN-62 and KN-93 exposure reduced hypoxia-mediated energy status signaling and glucose transport in fast-twitch skeletal muscle (3). Exercise-induced AMPK activation is associated largely with complexes containing the γ3-subunit (3), indicating that AMPKγ3-KO contraction-mediated glucose transport is unaffected (2, 11), suggesting that activation of other AMPKγ2- and γ3-containing heterotrimers may be dispensable for contraction-mediated glucose transport. In these same animal models (2, 11), glucose transport in response to AMPK activation by the adenosine analog 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) is completely ablated, suggesting that multiple non-insulin-mediated (energy-sensing) pathways regulate glucose transport. Whether hypoxia and contraction utilize similar or distinct isoform-specific AMPK-mediated pathways to promote glucose transport is unknown.

Dantrolene, an inhibitor of Ca2+ release from the sarcoplasmic reticulum, attenuates hypoxia-mediated glucose transport (5), providing evidence for an important role for Ca2+ in mediating energy status signaling. Ca2+/calmodulin (CaM)-competitive inhibitors of CaM-dependent protein kinase (CaMK) KN-62 and KN-93 have been used to determine the role of Ca2+ in contraction-mediated glucose transport (31, 32). Although these inhibitors block CaMK, they may also have off-target effects, since they have also been shown to block insulin-stimulated glucose uptake in rat soleus and epitroclearis muscle (30) and voltage-gated potassium channels in human embryonic kidney-293 cells (21). In slow-twitch rat soleus muscle, contraction and hypoxia-stimulated glucose uptake were completely dependent on Ca2+-dependent pathways (31), whereas in rat fast-twitch glycolytic muscle, AMPK and Ca2+-mediated pathways were linked to contraction-mediated glucose transport (32), suggesting a fiber-type dependency. Conversely, in skeletal muscle from transgenic mice overexpressing kinase-dead AMPKγ2, hypoxia-mediated glucose transport was completely abolished (20), suggesting that hypoxia-stimulated glucose transport is largely AMPK dependent.

SKELETAL MUSCLE GLUCOSE TRANSPORT can be regulated by separate and distinct signaling pathways. In skeletal muscle from type 2 diabetic patients, defects in the canonical insulin signal transduction cascade (17) are coupled to aberrant insulin-stimulated GLUT4 translocation and glucose transport (23). Changes in the energy status of the cell also elicit signaling cascades that promote glucose uptake in skeletal muscle (18). Exercise (13, 27), hypoxia (1, 23), or AMP-activated protein kinase (AMPK) activation (14) can bypass defects in insulin signaling in type 2 diabetes to promote GLUT4 translocation and glucose uptake. Signaling pathways involving AMPK and/or Ca2+ regulate GLUT4 translocation and glucose transport in an insulin-independent manner in skeletal muscle (4, 5, 10, 20, 31, 32) by incompletely described mechanisms. Activation of these energy status-signaling pathways may provide additional levels of regulation to control glucose homeostasis in insulin-resistant states.

AMPK is an energy sensor that is activated in response to changes in the AMP/ATP ratio associated with exercise/muscle contraction, cellular stress, or hypoxia (18). AMPK is a heterotrimeric complex consisting of three subunits with seven isoforms (α2, β2, and γ3). Of the resulting 12 possible heterotrimers, only three appear to be expressed in human skeletal muscle (3). Exercise-induced AMPK activation is associated largely with complexes containing the γ3-subunit (3), indicating that AMPKγ3-KO contraction-mediated glucose transport is unaffected (2, 11), suggesting that activation of other AMPKγ2- and γ3-containing heterotrimers may be dispensable for contraction-mediated glucose transport. In these same animal models (2, 11), glucose transport in response to AMPK activation by the adenosine analog 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) is completely ablated, suggesting that multiple non-insulin-mediated (energy-sensing) pathways regulate glucose transport. Whether hypoxia and contraction utilize similar or distinct isoform-specific AMPK-mediated pathways to promote glucose transport is unknown.

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We hypothesized that the AMPKγ3 isoform is necessary for hypoxia-stimulated glucose transport in glycolytic muscle. We provide direct evidence that the AMPKγ3 isoform plays a role in hypoxia-stimulated glucose transport in fast-twitch glycolytic skeletal muscle using AMPKγ3-KO mice. Furthermore, the Ca^2+/-calmodulin-competitive inhibitor KN-93 reduced hypoxia-mediated glucose transport.

**MATERIALS AND METHODS**

**Animals.** All experiments were approved by the Regional Animal Ethics Committee, Stockholm, Sweden. Female C57BL/6 mice, AMPKγ3-KO mice backcrossed to C57BL/6 background (2), and wild-type littermates (3–5 mo old) were used in this study. Mice were maintained on a 12:12-h light-dark cycle and received standard rodent chow. Mice were fasted 4 h prior to study.

**Muscle incubation.** Extensor digitorum longus (EDL) and soleus muscles were removed from anesthetized mice (99% 2,2,2-tribromoethanol and tertiary amyl alcohol at 0.015–0.017 ml/g mouse body wt ip; Avertin) and incubated for 30 min at 30°C in vials containing preoxygenated (95% O_2-5% CO_2) Krebs-Henseleit buffer (KHB) containing 5 mM HEPES (pH 7.0) and supplemented with 15 mM mannitol and 5 mM glucose. Muscles were then transferred to new vials containing fresh KHB pregassed either as described above (normoxia) or with 95% N_2-5% CO_2 (hypoxia) and incubated for 45 min. When specifically indicated, a selective Ca^2+/-CaMK inhibitor (KN-93, 25 g/ml; Sigma) or a pseudo inhibitor of Ca^2+/-CaMK (KN-92, 25 g/ml; Calbiochem) (30) was added to the medium of the incubation steps. Muscles were either immediately frozen in liquid nitrogen and stored at −80°C for subsequent signal transduction analysis or subjected to additional incubation as described below for glucose transport.

**Glucose transport.** Muscles were transferred to new vials containing preoxygenated KHB supplemented with 18 mM mannitol and 2 mM pyruvic acid and incubated for 15 min. Muscles were then transferred to new vials containing preoxygenated KHB supplemented with 1 mM 2-deoxy-[1,2-3H]glucose (2.5 gCi/ml) and 19 mM [1^4]C]mannitol and incubated for 20 min. Glucose transport was determined as described previously (7).

**Signal transduction.** Muscles were homogenized in ice-cold buffer [10% glycerol, 5 mM sodium pyrophosphate, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2, 20 mM Tris (pH 7.8), 1% Triton X-100, 10 mM sodium fluoride, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, 1 g/ml leupeptin, 0.5 mM sodium vanadate, 1 mM benzamidine, 1 mM microcystin] for 20 s using a motor-driven pestle. Homogenates were rotated end over end for 10 min) at 4°C. The supernatant was frozen in liquid nitrogen and stored at −80°C for subsequent signal transduction analysis or subjected to additional incubation as described below for glucose transport.

**Muscle lysate.** Muscles were transferred to new vials containing preoxygenated KHB supplemented with 18 mM mannitol and 2 mM pyruvic acid and incubated for 15 min. Muscles were then transferred to new vials containing preoxygenated KHB supplemented with 1 mM 2-deoxy-[1,2-3H]glucose (2.5 gCi/ml) and 19 mM [1^4]C]mannitol and incubated for 20 min. Glucose transport was determined as described previously (7).

**AMPK activity assay.** Skeletal muscle lysate (350 g of protein) was immunoprecipitated with 3 g of TBC1D4 antibody (Upstate Biotechnologies) at 4°C with gentle rotation over-

**Fig. 1.** Hypoxia-mediated glucose transport. A: extensor digitorum longus (EDL) muscle from C57BL/6 mice was incubated under normoxic or hypoxic conditions for 45 min. 2-Deoxyglucose transport (GT; nmol/g protein) was determined as means ± SE; n = 5–7 muscles. ** and *** P < 0.01 and 0.005, respectively, compared with basal; †P < 0.05 compared with hypoxia effect. B: soleus muscle from AMP-activated protein kinase (AMPK)γ3-knockout (KO) mice and wild-type (WT) littermates was incubated under normoxic or hypoxic conditions for 45 min. 2-Deoxyglucose transport is reported as means ± SE; n = 5–6 muscles. **P < 0.001 for treatment effect.
night. Samples were incubated with protein G-agarose (Sigma-Aldrich, St. Louis, MO) for 3 h at 4°C and subsequently washed three times with homogenization buffer and four times with phosphate-buffered saline. The immunoprecipitated and immunodepleted samples were boiled in Laemmli buffer containing β-mercaptoethanol and subjected to SDS-PAGE. Phosphorylation and total protein expression of AS160 were determined by using PAS and AS160 antibody.

**Statistics.** Data are expressed as means ± SE. Statistical evaluation was performed by one-way or two-way ANOVA, and Tukey’s post hoc analysis was applied to identify significant differences between groups when interaction effects were statistically significant. Levene’s test of equality was performed to ensure equality of variances between the groups. When significance was observed, data were log transformed. In all cases, log transformation was sufficient to obtain equal variances among groups. *P* < 0.05 was considered significant.

**RESULTS**

**Glucose transport.** Effect of KN-92 and KN-93 on basal and hypoxia-mediated 2-deoxyglucose transport was determined in EDL muscle from C57BL/6 mice (Fig. 1A). Basal glucose transport was comparable between KN-92- and KN-93-treated muscle. Hypoxia increased glucose transport 2.6-fold (*P* < 0.001). KN-93 treatment reduced (*P* < 0.05) hypoxia-mediated glucose transport, providing evidence to suggest that Ca²⁺/CaMKs may contribute to hypoxia-induced glucose transport. In contrast, KN-92 treatment was without effect on hypoxia-mediated glucose transport, which was expected since (KN-92) is a pseudo inhibitor of Ca²⁺/CaMK. Consequently, KN-92 was excluded from the remaining analysis. Basal and hypoxia-mediated 2-deoxyglucose transport was determined in soleus muscle from AMPKγ3-KO mice and wild-type littermates (Fig. 1B). Hypoxia increased glucose transport in soleus muscle, irrespective of the genotype. Basal and hypoxia-stimulated glucose transport were comparable between genotypes.
glucose transport was comparable between wild-type and AMPKγ3-KO mice (Fig. 2A). Hypoxia increased glucose transport in EDL muscle from wild-type mice \((P < 0.001)\). Hypoxia-mediated glucose transport was attenuated (45% reduction) in EDL muscle from AMPKγ3-KO mice \((P < 0.01)\). KN-93 significantly reduced \((P < 0.05)\) hypoxia-mediated glucose transport in EDL muscle from AMPKγ3-KO (16%) and wild-type (20%) mice (Fig. 2A). The level of statistical significance for this interaction is \(P = 0.009\).

**Signal transduction.** Phosphorylation of proteins implicated in hypoxia-mediated signaling toward glucose transport was assessed. Basal CaMKII and AMPK phosphorylation were comparable between genotypes (Fig. 2, B and C). Hypoxia increased the CaMKII and AMPK phosphorylation in AMPKγ3-KO mice and wild-type littersmates to a similar extent \((P < 0.001;\) Fig. 2, B and C). Hypoxia increased ACC phosphorylation 29-fold \((P < 0.001;\) Fig. 2D). Basal and hypoxia-stimulated ACC phosphorylation were comparable between genotypes, although a trend for a reduction in the hypoxia-stimulated response was observed in AMPKγ3-KO mice. The effect of hypoxia on CaMKII phosphorylation in AMPKγ3-KO and wild-type mice was attenuated in the presence of KN-93 \((P < 0.05;\) Fig. 2B), whereas phosphorylation of AMPK and ACC was unaltered. (Fig. 2, C and D). Hypoxia increased isoform-specific α1- and α2-associated AMPK activity in AMPKγ3-KO mice and wild-type littersmates to a similar extent, and this response was unaltered in the presence of KN-93. To further delineate the importance of signaling events toward glucose transport, TBC1D1/D4 phosphorylation was assessed using the PAS antibody. TBC1D4 (AS160) and TBC1D1 are Rab GTPase-activating proteins that are implicated in insulin-stimulated glucose transport in adipocytes (24) and are AMPK targets in mouse glycolytic EDL muscle (26, 28). Hypoxia exposure increased phosphorylation of TBC1D1/D4 in EDL muscle twofold \((P < 0.01;\) Fig. 3A). Basal and hypoxia-mediated TBC1D1/D4 phosphorylation was similar between the wild-type and AMPKγ3-KO mice. In KN-93-treated EDL muscle, hypoxia-induced TBC1D1/D4 phosphorylation was reduced in AMPKγ3-KO mice \((P < 0.05)\) and unaltered in wild-type mice (Fig. 3A). Since TBC1D4 and TBC1D1 are expressed in mouse EDL muscle (26), we attempted to differentiate phosphorylation of these individual proteins. Immunoprecipitation of mouse EDL muscle with TBC1D4, followed by immunoblot analysis with PAS (Fig. 3B), showed a phosphorylation pattern similar to the PAS immunoblot of muscle lysates (Fig. 3, A and C). Immunoblot analysis with TBC1D4 (Fig. 3B) reveals that...
this protein is expressed at low levels in EDL muscle. Since PAS signals were undetected in TBC1D4-immunodepleted samples (Fig. 3B), TBC1D4 is likely to be the major phosphorylated isoform in mouse EDL muscle (Fig. 3, A and C). Phosphorylation of Akt Ser^473 was unaltered during all incubation conditions (data not shown). Skeletal muscle GLUT4 protein expression was unaltered between wild-type and AMPK_3-KO mice (9.61 ± 0.72 vs. 9.9 ± 1.67, respectively; n = 8). AMPK, ACC, and CaMKII phosphorylation was normalized for expression level of each respective total protein (Fig. 3C).

**DISCUSSION**

In skeletal muscle, the canonical insulin-signaling cascade and energy-sensing pathways provide separate and independent mechanisms to increase glucose transport (8). Whereas the insulin-signaling cascade has been widely studied (25), comparatively less is known about the distinct energy-sensing pathways involved in glucose transport. Evidence is emerging to support the hypothesis that multiple energy-sensing pathways contribute to the regulation of glucose transport (9, 29). A further level of complexity is conferred by the expression of calcium- and AMP-dependent, isoform-specific signal transducers, which have been implicated in the regulation of glucose transport in response to a variety of energy-depleting stimuli, including exercise, AICAR, and hypoxia (18). Here, we provide evidence that the AMPK_3 isoform plays an important role in mediating hypoxia-stimulated glucose transport in glycolytic muscle. Our results also suggest that the AMPK_3 isoform is dispensable for the Ca^{2+}-dependent signaling inputs that contribute to the regulation of glucose transport in response to hypoxia.

The maximal effect of insulin and exercise/muscle contraction on glucose transport is additive, suggesting that two separate and distinct signaling pathways contribute to the regulation of glucose transport (9, 29). In contrast, exercise/muscle contraction and hypoxia do not elicit an additive effect on glucose transport, indicating that at some level a common signaling mechanism exists (1, 5). Although AMPK has been widely implicated in the regulation of glucose transport in response to changes in the cellular energy status (18), the evaluation of this target has been complex due to the multiple isoforms expressed. We focused on the role of the AMPK_3 isoform since this isoform is expressed predominantly in glycolytic skeletal muscle (19) and contains heterotrimers are activated in response to exercise (3). We have shown previously that the AMPK_3 isoform is necessary for the effect of AICAR to promote glucose transport in isolated fast-twitch EDL muscle (2). In contrast, the AMPK_3 isoform is dispensable for contraction-mediated glucose transport (2). Early studies provide evidence that calcium-mediated signaling pathways contribute to the effect of hypoxia on glucose transport (5). For example, exposure of rat epitrochlearis muscle to dantrolene, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum, partly prevented the effect of hypoxia on glucose transport (32). This would suggest that, in addition to AMPK pathways, Ca^{2+}-dependent pathways regulate glucose transport in response to hypoxia. AMPK- and Ca^{2+}-mediated signaling have also been linked to contraction-mediated glucose transport in rat fast-twitch glycolytic muscle (32).

Here, we provide evidence that hypoxia-mediated glucose transport is mediated via AMPK_3-dependent as well as AMPK_3-independent mechanisms, since glucose transport in EDL muscle was partly reduced in AMPK_3-KO mice. Conversely, in soleus muscle, where the AMPK_3 subunit expressed low but comparable levels between the genotypes (19), the effect of hypoxia on glucose transport was similar between genotypes. Our results also support the hypothesis that AMPK- and Ca^{2+}-dependent signaling inputs overlap, since the effect of KN-93 reduced hypoxia-mediated glucose transport in wild-type and AMPK_3-KO mice with concomitant reduction in phosphorylation of CaMKII, suggesting that AMPK- and Ca^{2+}-mediated pathways regulate glucose transport under these conditions. In contrast, hypoxia-mediated glucose transport was completely prevented in AMPK_2 kinase-dead mice, implicating AMPK as the primary regulator of hypoxia-induced glucose transport (20). A simplistic view is that Ca^{2+}-dependent kinases and AMPK control separate linear signaling pathways. Alternatively, Ca^{2+}-dependent kinases may lie upstream from AMPK (10). Indeed, CaMK kinase regulates AMPK phosphorylation and glucose uptake in mouse skeletal muscle at the onset of tetanic contraction in an intensity- and/or time-dependent manner (10).

To further elucidate signaling pathways contributing to hypoxia-mediated glucose transport, phosphorylation of AMPK, Akt, and the downstream targets ACC and TBC1D1/D4 was determined. Exposure to hypoxia increased AMPK phosphorylation in EDL muscle from wild-type and AMPK_3-KO mice to a similar extent, with a parallel increase in ACC phosphorylation. The effect of KN-93 on AMPK and ACC phosphorylation was comparable between wild-type and AMPK_3-KO mice. Hypoxia increased isoform-specific α1- and α2-associated AMPK activity in EDL muscle irrespective of genotype, and this effect was unaltered by KN-93 treatment, suggesting that hypoxia-mediated AMPK activation is independent of Ca^{2+}-mediated signaling inputs. These results also provide evidence that the AMPK_3 isoform is dispensable for hypoxia-induced AMPK activation and are consistent with previous studies (Fig. 4) showing that AICAR-mediated AMPK and

![Fig. 4. Overview of the regulation of glucose transport and key signaling events in EDL muscle from AMPK_3-KO mice in response to 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), contraction, and hypoxia based on present and previous results (2, 28).](http://ajpendo.physiology.org/)
ACC phosphorylation is maintained despite a profound impairment in glucose transport (2). However, at earlier time points, AICAR-mediated AMPK phosphorylation is reduced in AMPKα3-KO mice (28).

TBC1D1 and TBC1D4/AS160 are Rab GTPase-activating proteins involved in glucose transport in adipocytes (24) and skeletal muscle (16, 26). In mouse glycolytic skeletal muscle, the AMPKγ3 isoform is necessary for AICAR-stimulated TBC1D4 (AS160) phosphorylation but dispensable for the contraction-mediated response (28). Hypoxia-mediated TBC1D1/D4 phosphorylation was similar between wild-type and AMPKγ3-KO mice. Because signals detected using the PAS antibody may reflect phosphorylation of TBC1D1, an AS160-related protein that regulates GLUT4 translocation (22) and lipid oxidation (6) in skeletal muscle, we attempted to differentiate phosphorylation of TBC1D1 and TBC1D4. Our results indicate that TBC1D4 plays a role in hypoxia-mediated glucose transport. However, we cannot exclude a possible role of TBC1D1, particularly because this isoform is expressed at high levels in EDL muscle (26) and is known to be regulated by AMPK (6). Thus, our results suggest that hypoxia-mediated TBC1D1/D4/D4 phosphorylation is uncoupled from glucose transport in AMPKγ3-KO mice, similar to earlier reports for contraction-mediated events in mice with impaired AMPK signaling (28). This provides evidence that TBC1D1/D4-independent mechanisms contribute to hypoxia- as well as contraction-mediated glucose transport in skeletal muscle. Interestingly, KN-93 exposure prevented the effect of hypoxia on TBC1D1/D4 phosphorylation in AMPKγ3-KO but not wild-type mice. This result suggests that Ca²⁺-dependent signaling pathways may be altered from an adaptive response arising from AMPKγ3 depletion. Since TBC1D4 has a CaM-binding site (12, 15), KN-93 may inhibit this site directly and prevent TBC1D4 phosphorylation (15), highlighting a possible role of calcium-mediated events in TBC1D1/D4 phosphorylation. We cannot exclude the possibility that other unknown kinases in addition to AMPK and CaMK are involved in hypoxia-induced TBC1D1/D4 phosphorylation.

The diverse role of the AMPKγ3 isoform in contraction-, AICAR-, and hypoxia-mediated glucose transport provides further evidence for the complexity in the signaling pathways regulating glucose transport in skeletal muscle (Fig. 4). Collectively, our results suggest that AMPKγ3 isoform couples the stimulation of glucose transport, in response to AICAR or hypoxia, but not contraction. We cannot exclude the possibility that other AMPKγ isoforms contribute to the regulation of glucose transport in response to hypoxia. For example, transgenic overexpression of a kinase-dead mutant form of AMPKα2 in skeletal muscle completely prevented hypoxia-stimulated glucose transport (20). In this model, the AMPK catalytic subunit is inactivated, and consequently, the specific activity associated with all γ-isoforms is ablated. This would suggest that, in addition to γ3-containing heterotrimers, other γ-isoform-containing heterotrimers contribute to hypoxia-mediated glucose transport.

In conclusion, we provide direct evidence for a role of AMPKγ3 in hypoxia-mediated glucose transport in isolated fast-twitch glycolytic skeletal muscle. Our data support an additional role of Ca²⁺-mediated signaling events in hypoxia-induced glucose transport. Furthermore, hypoxia-mediated glucose transport is uncoupled from TBC1D1/D4 phosphorylation in the absence of the AMPKγ3 isoform, indicating that TBC1D1/D4-independent mechanisms contribute to glucose transport in skeletal muscle.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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