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Original article

Neuronal nitric oxide synthase mediates insulin- and oxidative stress-induced glucose uptake in skeletal muscle myotubes

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ABSTRACT

Previously published studies strongly suggested that insulin- and exercise-induced skeletal muscle glucose uptake require nitric oxide (NO) production. However, the signal transduction mechanisms by which insulin and contraction regulated NO production and subsequent glucose transport are not known. In the present study, we utilized the myotube cell lines treated with insulin or hydrogen peroxide, the latter to mimic contraction-induced oxidative stress, to characterize these mechanisms. We found that insulin stimulation of neuronal nitric oxide synthase (nNOS) phosphorylation, NO production, and GLUT4 translocation were all significantly reduced by inhibition of either nNOS or Akt2. Hydrogen peroxide (H_2O_2) induced phosphorylation of nNOS at the same residue as did insulin, and also stimulated NO production and GLUT4 translocation. nNOS inhibition prevented H_2O_2-induced GLUT4 translocation. AMP activated protein kinase (AMPK) inhibition prevented H_2O_2 activation and phosphorylation of nNOS, leading to reduced NO production and significantly attenuated GLUT4 translocation. We conclude that nNOS phosphorylation and subsequently increased NO production are required for both insulin- and H_2O_2-stimulated glucose transport. Although the two stimuli result in phosphorylation of the same residue on nNOS, they do so through distinct protein kinases. Thus, insulin and H_2O_2-activated signaling pathways converge on nNOS, which is a common mediator of glucose uptake in both pathways. However, the fact that different kinases are utilized provides a basis for the use of exercise to activate glucose transport in the face of insulin resistance.

1. Introduction

Nitric Oxide (NO) plays a critical role in skeletal muscle (SkM) physiology [1–3] and has been implicated in glucose uptake during exercise [4–9] and in response to insulin [8,10,11]. However, the regulatory mechanisms controlling endogenous NO production by nitric oxide synthases (NOSs) in SkM are not fully understood. The dominant NOS in SkM is a splice variant of nNOS, termed nNOSμ, which is present only in skeletal and cardiac muscle [12].

The physiological importance of NO is highlighted by reduced basal and insulin-stimulated NO synthesis in the SkM [10] of type 2 diabetics (T2DM). Coupled with studies that NOS KO mice are insulin resistant [13], this suggests that insulin action in SkM requires NO production and that impaired NO synthesis contributes to insulin resistance in T2DM. Studies in our laboratory demonstrating that nNOSμ is phosphorylated in a key intrinsic regulatory region at S1446 in response to insulin treatment [14] suggest that the molecular mechanism for the response to insulin seen in the human studies involves phosphorylation of S1446. Protein kinase B (Akt) is an established signal transduction kinase that controls cellular energy homeostasis [15] and could be responsible for signal transduction controlling NO production through phosphorylation of S1446.

Both insulin and exercise are potent inducers of glucose uptake through translocation of glucose transporter type 4 (GLUT4) from its intracellular location to the plasma membrane. However, exercise is thought to induce GLUT4 translocation through a separate mechanism regulated by AMP activated protein kinase (AMPK), rather than Akt. This is evidenced by intact exercise-stimulated glucose uptake despite a deficient insulin response in T2DM [16,17]. Hydrogen peroxide (H_2O_2) is generated in skeletal muscle during exercise [18–21] has been suggested to be a mediator of exercise-induced glucose uptake [22–25]. AMPK is acutely activated during exercise through oxidative stress (OS)

Abbreviations: NOS, nitric oxide synthase; NO, nitric oxide; Akt, protein kinase B; AMPK, AMP activated protein kinase; SkM, skeletal muscle; T2DM, type 2 diabetics; GLUT4, glucose transporter type 4; H_2O_2, hydrogen peroxide; Dsm, dorsomorphin

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[26,27], and studies from our laboratory demonstrated that H_2O_2 treatment caused AMPK-mediated nNOS phosphorylation in a cardiomyocyte cell line [28]. Directly testing the effects of exogenous H_2O_2 on isolated C2C12 and L6 myotube cell lines reduces the confounding variables and allows characterization of the SkM-specific mechanisms controlling NO production and glucose uptake. Moreover, C2C12 myotubes have been previously used to understand SkM responses to oxidative stress and contraction [26,29,30] and are considered good models for cellular responses to exercise. We therefore selected this *in vitro* model to study the role of NO in exercise-mediated glucose uptake.

The aim of this study is to test the hypothesis that glucose uptake stimulated by both insulin and exercise is mediated by NO production from nNOSμ. In the current study, we have utilized cell culture models to examine whether insulin and oxidative stress, which is associated with exercise, promote nNOS phosphorylation, GLUT4 translocation, and glucose uptake in a manner that is dependent on the presence of nNOSμ and on NO synthesis. We demonstrate that both insulin and oxidative stress activate nNOSμ *via* phosphorylation by Akt and AMPK, respectively, and that this increase in NO is necessary for subsequent GLUT4 translocation. Understanding the mechanisms by which oxidative stress and insulin promote muscle glucose uptake is essential for understanding exercise physiology and metabolic disease [31–34], and developing effective therapies to treat insulin resistance.

2. Materials and methods

2.1. Reagents and cell culture

For studies using H_2O_2, C2C12 myoblast cells (ATCC) were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose (Gibco), 1% penicillin-streptomycin (Gibco), 2 mM L-glutamine (Gibco) with 10% fetal bovine serum (Gibco). For studies using insulin, C2C12 myoblasts were cultured under the same conditions as above, except that media contained 1 g/L glucose. C2C12 myoblasts were cultured in growth media to confluence then differentiated to myotubes in differentiation media (DMEM as above but with 2% horse serum) for 5 days before experimentation. Myotubes were then treated with insulin, (100–200 nM; Sigma-Aldrich), H_2O_2 (400 μM, or as indicated in Figure Legends; Sigma-Aldrich), 20 μM Dorsomorphin (AMPKα inhibitor; Santa Cruz Biotechnology), 10 μM Akti-2 (Akt2 inhibitor; Calbiochem), 3 mM L-NAME (NOS inhibitor; Sigma-Aldrich), or 5 μM gp91 ds-tat (NOX2 inhibitor; AnaSpec, Inc), as indicated in each figure.

2.2. NO detection

C2C12 myoblasts were plated and differentiated in clear bottom 12 or 24 well plates (Corning) over 5 days (8 biologic replicates). Myotubes were incubated in serum- and phenol red-free media for 1 h prior to the beginning of the experiment. Myotubes were then incubated with 5 μM 4,5-diaminofluorescein-diacetate (DAF- 2-DA; Enzo) for 15 min prior to the addition of 100 nM insulin or 400 μM H_2O_2. If inhibitors were used in the experiment, they were added for 30 min before insulin or H_2O_2 treatment. After treatment, cells were washed with PBS to remove excess DAF-2-DA. Fluorescent and phase contrast images (20×) were obtained using an Olympus IX70 fluorescence microscope (Em_{max}: 515 nm), using an exposure time based on control conditions. Four high-powered fields were imaged per well (32 total replicates per experimental setting). Every experimental image with DAF-2-DA was adjusted with blank-field and background fluorescence (C2C12 myotubes without DAF-2-DA) and mean fluorescence intensity was calculated with ImageJ [35].

2.3. Immunoblot analysis

Whole cell lysates were collected from C2C12 cells in 2x Laemmeli buffer (0.125 M Tris HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) containing 1x benzonase nuclease (Sigma-Aldrich), protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were subjected to PAGE (Mini-PROTEAN TGX 4–15% gels; Biorad), transferred onto PVDF membranes with Trans-Blot Turbo Transfer System (Biorad), then probed with the indicated antibodies using the iBind Flex Western Device (Thermo Fisher Scientific). Primary antibodies included phospho-S1417 nNOS, phospho-S473 Akt, total Akt, phospho-S153 AMPK, AMPKα, phospho-ACC, GAPDH (Cell Signaling Technology), and nNOS (developed in-house). Correct identification of the protein of interest was confirmed by molecular weight (ACC: 265 kDa, nNOS: 160 kDa, AMPK: 63 kDa, Akt: 63 kDa, GAPDH: 36 kDa). Goat HRP-conjugated secondary anti-rabbit secondary antibody was from Santa Cruz. Blots were developed using the Immobilon western chemiluminiscent substrate (Merck Millipore, Billerica, MA), imaged with a CCD camera or blue X-ray film (Phenix), and analyzed using ImageJ [35] or Image Studio Lite (LI-COR).

2.4. GLUT4-myc immunohistochemistry

Translocation of the GLUT4 receptor from intracellular storage to the plasma membrane was monitored using L6 muscle cells that stably express myc-tagged GLUT4myc (L6-GLUT4myc cells; Kerafast), as described [36], with some minor changes. Myoblasts were plated on poly-D-lysine-coated coverslips (Neuvitro) and differentiated using DMEM with 2% horse serum over 5 days prior to experimentation. Myotubes were preincubated in serum- and phenol red-free media for 1 h prior to the beginning of the experiment. Cells were then pretreated for 30 min with 20 μM Dorsomorphin, 20 μM Akt2i or 3 mM L-NAME, followed by treatment with 200 nM insulin or 400 μM H_2O_2 for 30 min. Cells were fixed with 4% paraformaldehyde in PBS, and immunostaining for c-myc (Sigma-Aldrich) and Cy3-labeled 2° antibody (Jackson ImmunoResearch Laboratories) was performed as described [37]. Nuclear counterstaining was with 200 nM DAPI (ThermoFisher) before mounting coverslips using Prolong gold media (ThermoFisher). Images were acquired using exposure times based on control conditions for Cy3 fluorescence (between 833 and 1200 ms). Background fluorescence was assessed with coverslips not treated with Cy3-labeled secondary antibodies and subtracted from all experimental images. Cy3 and DAPI fluorescent channels were merged and overlaid on phase contrast images using ImageJ [35].

2.5. Glucose uptake assay

C2C12 myotubes, grown in 6-well plates, were incubated in Krebs-Henseleit buffer without glucose and with or without L-NAME for 60 min prior to the start of the experiment. Cells were treated with vehicle or insulin (100 nM) for 10 min followed by incubation with 10 μM 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG; Cayman Chemical) for 1 h. Cells were washed 3x with ice cold PBS, then lysed with 0.2 N NaOH. Fluorescence of the supernatants was measured (Excitation/emission = 485/535 nm). Significance was measured by student *t*-test, unpaired data with unequal variance.

2.6. Statistics

All experimental values are expressed as means ± standard error of the mean (SEM) of at least 3 biological replicates. Comparisons were performed using students *t*-test, unpaired data with unequal variance, using the software KaleidaGraph (Synergy Software) or www.graphpad.com. Statistical significance threshold is $p < 0.05$ compared to control conditions or as indicated in the figure legends.

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