Protein Kinase (AMPK) and PGC-1α in Skeletal Muscle

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Skeletal muscle is the major site of insulin-stimulated glucose uptake and oxidation processes which are controlled, in part, by activation of the cellular energy-sensor, AMP-activated protein kinase (AMPK). In addition, the transcription coactivator, peroxisome-proliferator activated receptor γ coactivator-1α (PGC-1α), has been considered to be a master regulator of cellular metabolism. Recently, nitric oxide (NO) was shown to be involved in mitochondrial biogenesis in skeletal muscle. Here we aim to find whether NO up-regulates PGC-1α mRNA expression in L6 myotubes and test whether the AMPK-dependent up-regulation of PGC-1α and mitochondrial genes are influenced by nitric oxide synthase (NOS) activity. METHODS: Rat L6 myotubes were differentiated by serum withdrawal to form confluent myotube cultures. Differentiated myotubes were exposed to various treatments and harvested for measurement of specific mRNAs via RT-real time PCR. RESULTS: Diethylenetriamine NONOate (DETA-NO; 50 µM) increased PGC-1α mRNA expression after 3 hr. This was a transient effect, returning to baseline at 6 hrs. Treatment with the AMPK activating compound, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; 3 mM) increased phospho-to-total (α) AMPK ratio, and expression of mRNAs for PGC-1α, F1 ATP synthase, and citrate synthase, while co-treatment of myotubes with L-Nitroarginine methyl ester (L-NAME; 100µM), prevented these effects. CONCLUSIONS: NO is sufficient to induce PGC-1α mRNA, and NOS activity is required for AMPK activation, and induction of PGC-1α and mitochondrial gene expression.

Introduction

Insulin is the primary anabolic hormone that stimulates uptake and storage of fuel substances in skeletal muscle3. Type 2 diabetes is a metabolic disorder in which there is insulin resistance in peripheral tissues. It is well known that exercise helps control diabetes by increasing insulin sensitivity and decreasing adiposity. In fact, skeletal muscle accounts for 65% to 90% of the clearance of an oral or intravenous glucose challenge5. Regular physical activity increases the capacity of skeletal muscle for respiration, oxidation, energy expenditure, and up take of glucose in part by the increase in content of GLUT 4 and mitochondrial biogenesis8.

Accumulating evidence suggest that the enzyme AMP-activated protein kinase (AMPK), which is stimulated upon increases in AMP/ATP ratio, plays an important role in several metabolic processes during exercise11. AMPK activity increases in the setting of increased intracellular AMP and decreased creatine phosphate and is thought to act as a metabolic stress protein10. Further, the peroxisome-proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α) is emerging as the master regulator of mitochondrial biogenesis13. Previous studies suggest a link between these two molecules since activation of AMPK increases PGC-1α expression and mitochondrial biogenesis and forced expression of a dominant negative form of AMPK can block these changes in skeletal muscle5,6.

Nitric Oxide (NO) is a ubiquitous signaling molecule involved in various physiological functions. NO is produced enzymatically from nitric oxide synthases, and its synthesis increases during contraction. Skeletal muscle expresses neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) NOS isoforms4. eNOS and nNOS synthesize NO at lower levels, whereas iNOS expression increases during inflammation and acute exercise and induces much higher NO production4. Some of the physiological and pathological effects of NO result from its actions at the mitochondrial level7. Nisoli et al. (2003) reported that NO-induced mitochondrial biogenesis required induction of PGC-1α expression in brown adipose tissue and also that eNOS−/− mice have decreased PGC-1α mRNA levels and reduced mitochondrial density. In addition, Fryer et al.2 suggested a positive feedback interaction between AMPK and NOS in skeletal muscle.

Our lab has recently reported that NO increases GLUT 4 expression and regulates AMPK signaling in skeletal muscle. In this study we aim to find whether NO up-regulates PGC-1α mRNA expression in L6 skeletal muscle myotubes and test whether the AMPK-dependent up-regulation of PGC-1α and mitochondrial genes (F1 ATP Synthase [F1ATP] and Citrate Synthase [CS]) are influenced by NOS activity. Therefore we hypothesize that: 1) treatment with the NOS-inhibitor, N(G)-L-nitro-arginine methyl ester (L-NAME), will prevent the AMP-mimetic drug, AICAR-induced phosphorylation of AMPK and the AMPK-induced up-regulation of PGC-1α, F1ATP, and CS.
mRNAs, and 2) treatment of L6 myotubes with NO-donor, DETA-NO, will induce up-regulation of PGC-1α gene expression.

Methods

Chemicals.
AICAR was obtained from Toronto Research Chemicals (North York, ON). NG-nitro-L-arginine methyl ester (L-NAME), and S-nitroso-N-penicillamine (SNAP) were purchased from Cayman Chemical (Ann Arbor, MI). Compound C was obtained from Calbiochem (San Diego, CA) and diethylamine-NO (DETA-NO) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture.
Rat L6 myoblast were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37 °C in 5% CO₂ and 95% atmospheric air on 6-well collagen-coated plates. Myoblasts were grown in 10% Fetal Bovine Serum (FBS) medium containing 5 mM low glucose Dulbecco’s Modified Eagle’s Medium (DMEM), 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 6 days before treatment initiation.

Experimental Design.
AMPK activation experiments
Differentiated myotube cultures were treated for 1h with different concentrations of 5-aminoimidazole-4-carboxamide-1β-D-ribofuranoside (AICAR) [1-4 mM], N-nitro-L-arginine methyl ester (L-NAME) [100μM], and the AMPK inhibitor, Compound C (45μM). After treatment, cells were washed two times in ice-cold PBS containing 1 μM Na₃VO₄, 0.05% vol/vol protease inhibitors from Sigma (St. Louis, MO). Phospho-to-total (α) AMPK ratio was used as a dependent variable.

Gene expression experiments.
Experiment 1. Myotube cultures were treated for 16h with AICAR (1mM) and/or L-NAME (100μM) in treatment medium containing 10% HoS and 5% FBS. Enzyme inhibitors (L-NAME and Compound C) were added 30 min before other treatments. Following treatment, cells were washed two times in ice-cold PBS containing 1 μM Na₃VO₄, and immediately harvested in TRIzol Reagent (Invitrogen).

Experiment 2. Cells were exposed for either 3h or 6h to different doses (0 µM, 5 µM, 20 µM, and 50 µM) of the slow-release NO donor, diethylamine-NO (DETA-NO; half life of 20 hrs at 37°C, pH 7.4). Quantitative RT-real time PCR served to quantify changes in PGC-1α, F₁ ATP, CS. Treatment medium contained serum (10% HoS and 5% FBS) and one of the chemicals mentioned above following the same protocol as experiment 1.

Reverse Transcriptase and Real-Time PCR.
Total RNA was reverse transcribed using oligo (dT) 20 primers. One microliter of cDNA reaction mixture was added to a 25 μL PCR reaction for real-time PCR using Taqman chemistry and the ABI Prism 7000 Sequence Detection System. Relative quantitation of PGC-1α, F₁ ATP, and CS expression was performed using the comparative CT method. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as the normalizer. Primer and probe sequences for PGC-1α, F₁ ATP, and CS are commercially available from Applied Biosystems.

AMPK/ p-AMPK Western Blots.
Aliquots of cell lysates containing 30 μg of protein were run in a 10% SDS-PAGE gel, and then transferred to PVDF membranes (Millipore). Rabbit anti-(α)AMPK and anti-phospho(α)AMPK (1:1000 dilution, Cell Signaling) were used as primary antibodies, and protein levels were determined by densitometry (Kodak 1D Image Analysis Software version 3.6).

Statistical Analysis.
Results were treated with One-way ANOVA followed by the Fisher LSD test. Statistical significance was set a priori at P<0.05.

Results

Endogenous NO is required for AICAR-mediated AMPK activation and up-regulation of metabolic gene expression.
Treatment of L6 myotubes with the AMPK-activating drug AICAR (3 mM) showed a significant increased in Phospho-to-total (α) AMPK ratio. Co-treatment with Compound C or L-NAME prevented the AICAR-induced increase in AMPK phosphorylation (Fig. 1). Treatment of L6 myotubes with 1 mM AICAR increased expression of PGC-1α, F₁ ATP synthase, and citrate synthase mRNAs. Co-treatment with 100 μM L-NAME statistically prevented this effect (Fig. 2).

NO is sufficient to induce PGC-1α mRNA accumulation in skeletal muscle cells.
Treatment of myotubes with DETA-NO (50 µM) increased PGC-1α mRNA levels after 3 hrs. However, the effect was transient, decreasing to baseline after 6 hrs of treatment (Fig. 3).
Discussion

Our findings provide evidence that nitric oxide is sufficient to induce PGC-1α mRNA expression in skeletal muscle cells, and that endogenous NOS activity is required for AMPK-induced expression of PGC-1α and the mitochondrial genes, F1ATP synthase and citrate synthase. Several authors have identified NO as an important regulator of metabolic phenotype in skeletal muscle cells. The present data confirms that NO is a critical signal mediating energy-dependent regulation of AMPK, and that NO is capable of driving expression of the transcription co-activator, PGC-1α.

PGC-1α was first described and cloned in brown adipose tissue, but has since been found to play a central role in determination of skeletal muscle metabolic phenotype. PGC-1α does not bind to DNA directly, but interacts with, and enhances the action of transcription factors such as NRF-1 and MEF-2. Expression of PGC-1α in skeletal muscle is highest in slow-oxidative fibers, and is induced by exercise training. This suggests that PGC-1α action could drive oxidative adaptations, a notion that is confirmed by the observation of enhanced mitochondrial volume in the muscle of transgenic mice overexpressing PGC-1α.

PGC-1α expression is transcriptionally regulated via binding of myocyte enhancing factor (MEF) 2 and activating transcription factor (ATF) 2 to the PGC-1α promoter. This is very responsive to contractile and metabolic activity level of the muscle through a signaling pathway involving AMPK activation of p38 MAP kinase. Lira et al. recently reported that NO mediates GLUT4 expression in cultured myotubes via activation of AMPK. Further, endogenous NOS activity was required for AICAR-induction of GLUT4 mRNA and protein expression. This led to our hypothesis that NO-effects on skeletal muscle phenotype are mediated by activation of AMPK.

We report that inhibition of NOS by L-NAME prevents the effects of AICAR (an AMP mimetic drug) on PGC-1α and mitochondrial gene mRNAs. Further, L-NAME prevents AICAR-induced AMPK phosphorylation. These data suggest NO directly affects AMPK activation, most likely by activation of AMPK kinases such as LKB1, or inhibition of AMPK phosphatases. We also show that the NO donor, DETA-NO, is sufficient to induce PGC-1α mRNA. Lira et al. demonstrated that another NO donor, SNAP, was sufficient to induce AMPK activation. Therefore, NO is a major activator of the AMPK signaling pathway, independent of cellular energy status. Fig. 4 illustrates a proposed model of NO-dependent regulation of AMPK, leading to enhanced expression of PGC-1α and subsequent augmentation of cellular oxidative capacity.
Fig. 4. Proposed model of interaction between NO, PGC-1α, and AMPK in skeletal muscle. Our results suggest that NOS activity is required for AMPK activation and PGC-1α expression, both leading to increased mitochondrial biogenesis.

Significance

Skeletal muscle is the major site of insulin-stimulated glucose disposal, and insulin resistance. Exercise training effectively improves insulin sensitivity and metabolic control. Exercise-induced skeletal muscle adaptations responsible for this effect are mediated in part by activation of the cellular energy sensor, AMPK. Based on the known effects of AMPK on glucose metabolism and mitochondrial biogenesis in skeletal muscle it is clear that this molecule is a major player in the development and possible treatment of obesity, diabetes, and the metabolic syndrome X, which has now become an epidemic in modern industrialized countries. In addition to the acute metabolic functions of AMPK, AMPK also regulates expression of specific genes such as PGC-1α. The proposed effect NO has on AMPK and PGC-1α represents fruitful areas of further investigation. If this effect is shown to be present in humans then, it is possible that new NO donor compounds have potential clinical application.

References