Reactive Oxygen Species and p38 Mitogen-activated Protein Kinase Mediate Exercise-induced Skeletal Muscle-derived Interleukin-6 Expression

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Interleukin-6 (IL-6) is a pleiotropic cytokine secreted by many different cell types, and skeletal muscle is an important source of IL-6 during exercise. Here, we studied the effects of glucose deprivation *in vitro* on skeletal muscle-derived IL-6 expression and release in C2C12 myocytes, as well as its regulation by p38 mitogen-activated protein kinase (p38MAPK) and reactive oxygen species (ROS). C2C12 myotubes were cultured in DMEM medium containing 4.5 $g \cdot L^{-1}$ glucose (glucose control, GC) or DMEM medium containing no glucose (glucose deprivation, GD) for 0, 6, 12, 18 and 24 hours, and then incubated with 10 mM NAC (a ROS scavenger) or 10 μM SB203580 (a p38MAPK inhibitor) under either GC or GD conditions for 24 hours. IL-6 expression levels were subsequently analyzed using RT–PCR, and IL-6 protein levels in the medium were measured using ELISA. Glucose deprivation significantly enhanced IL-6 expression at 18 and 24 hours compared to the glucose control, and caused IL-6 protein levels to increase significantly over the entire 24-hour measurement period. The ROS scavenger NAC inhibited the glucose deprivation-induced release of IL-6 protein almost completely, while the p38MAPK inhibitor SB203580 inhibited glucose deprivation-induced IL-6 protein release to a lesser extent. Our study suggests that glucose deprivation in C2C12 myocytes induces IL-6 expression and release, and that this IL-6 release is mainly mediated via ROS signaling. Skeletal muscle-derived IL-6 may thus play an important role in energy metabolism during exercise. [*J Exerc Sci Fit* • Vol 9 • No 2 • 123–127 • 2011]

Keywords: C2C12, glucose deprivation, IL-6, p38MAPK, ROS

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine secreted by many different cell types, and recent studies have shown that it is linked to obesity and insulin resistance (Allen & Febbraio 2010). For example, IL-6-deficient mice developed mature-onset obesity and insulin resistance (Klover et al. 2005; Wallenius et al. 2002). Previous studies have suggested that IL-6 plays a role in the regulation of glucose homeostasis and the enhancement of lipid metabolism (Pedersen 2011; Al-Khalili et al. 2006; Carey et al. 2006; Klover et al. 2005; Petersen et al. 2005), and many studies have shown that skeletal muscle is an important source of IL-6. Coll et al. found that fatty acids, especially palmitate, can induce muscle myocyte IL-6 expression and release *in vitro* (Coll et al. 2008; Jove et al. 2005; Weigert et al. 2004), and Pedersen and colleagues demonstrated that IL-6 expression rapidly increases when resting human skeletal muscle is subjected to exercise (Pedersen et al. 2001; Steensberg et al. 2000). Exercise-induced IL-6 expression and release is influenced by muscle energy availability, and some studies have demonstrated that glucose ingestion during

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exercise attenuates exercise-induced increases in IL-6 plasma levels (Li et al. 2004; Lancaster et al. 2003; Nieman et al. 2003). Other studies have shown that muscle-derived IL-6 release increases during contractions when muscle glycogen levels are low (Keller et al. 2001; Steensberg et al. 2001). Experiments from our laboratory have indicated that low pre-exercise muscle glycogen content accelerates IL-6 expression in muscles and IL-6 protein increases in the plasma (Tang et al. 2007). While previous studies have highlighted the negative correlation between IL-6 expression and muscle glycogen content during exercise *in vivo*, there are no studies to date that have examined the effects of glucose deprivation on muscle-derived IL-6 expression and protein release in skeletal muscle cell cultures *in vitro*.

Recently, fragmentary data have suggested that both reactive oxygen species (ROS) and p38 mitogen-activated protein kinase (p38MAPK) may play a role in regulating IL-6 expression. Fischer et al. (2004) have shown that supplementation with the ROS scavengers vitamins C and E inhibits the release of IL-6 from contracting human skeletal muscle, while Sano et al. (2001) reported that the p38MAPK pathway is also critically involved in ROS-mediated induction of IL-6 release by angiotensin II in cardiac fibroblasts.

The purpose of this study was to test our hypothesis that glucose deprivation enhances IL-6 expression and protein release in C2C12 myocytes, and that this enhancement is regulated via ROS signaling and the p38MAPK pathway.

Methods

Cell culture

Mouse C2C12 myoblasts (Cell Center, Chinese Academy of Medical Sciences, China) were maintained in 12-well plates on Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, $100\,U \cdot mL^{-1}$ penicillin, and $100\,\mu g \cdot mL^{-1}$ streptomycin. When cells reached confluence, the medium was switched to a differentiation medium containing DMEM and 2% horse serum, which was changed daily. The differentiated C2C12 cells fused into myotubes 8 days after confluence.

Glucose deprivation induces IL-6 expression and release

Differentiated C2C12 myotubes were placed in DMEM medium containing $4.5\,g \cdot L^{-1}$ glucose (glucose control, GC) or DMEM medium containing no glucose (glucose deprivation, GD). Samples were incubated for 0, 6, 12, 18 and 24 hours, to measure IL-6 expression and protein release over time. Following incubation, IL-6 mRNA levels were analyzed using reverse-transcription–polymerase chain reaction (RT–PCR), and IL-6 protein levels in the medium were measured using ELISA.

Inhibition of ROS and the p38MAPK pathway

C2C12 myotubes were incubated with 10 mM N-acetyl-L-cysteine (NAC; Sigma-Aldrich, St. Louis, MO, USA), the precursor of the ROS scavenger glutathione, or 10 μM SB203580 (Merck, Darmstadt, Germany), a p38MAPK inhibitor, or alone (control) for 30 minutes. The myotubes were then incubated in DMEM medium containing $4.5\,g \cdot L^{-1}$ glucose (glucose control, GC) or DMEM medium containing no glucose (glucose deprivation, GD). There were six treatment groups in this experiment: GC; GD; GC+NAC; GD+NAC; GC+SB203580; and GD+SB203580. Following incubation, IL-6 protein levels in the medium were measured using ELISA.

RT–PCR

IL-6 expression in C2C12 myotubes was analyzed using RT–PCR. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. RNA concentrations were estimated by measuring absorbance at 260 nm, and purity was assessed by 260 nm/280 nm absorbance ratios (GeneQuant, Amersham Biosciences, Sweden). Denaturation of 1.5 μg total RNA and 2 μM oligo dT was carried out at 70°C for 5 minutes, immediately cooled, and then reverse transcribed using 4U M-MLV (Takara Bio, Otsu, Japan), 2U RNase Inhibitor, 0.4 mM dNTPs, and RT Buffer in a total volume of 25 μL. The reaction was reverse-transcribed at 42°C for 60 minutes and at 70°C for 15 minutes. RT–PCR was performed in a fluorescence temperature cycler (Rotor-Gene 6000, Corbett, Australia), containing 10 μL SYBR PCR Master Mix (Qiagen, Hilden, Germany), 0.5 μM Forward Primer, 0.5 μM Reverse Primer, and 5 ng cDNA in a total volume of 20 μL. Amplification involved a three-step cycle repeated 45 times after pre-incubation at 95°C for 10 minutes: denaturation at 94°C for 20 seconds; annealing at 58°C for 20 seconds; and extension at 72°C for 20 seconds. Primer pairs for the RT–PCR were, for the IL-6 gene (70 bp): 5′–CTGCAAGAGACTTCCATCCAGTT–3′ (forward), 5′–GAAGTAGGGAAGGCCGTGG–3′ (reverse); and for the β-actin gene (288 bp): 5′–GCTACAGCTTCACCACCACAG–3′ (forward), 5′–GGTCTTTACGGATGTCAACGTC–3′ (reverse). IL-6 mRNA abundance was normalized to that of β-actin.

ELISA

The concentration of IL-6 protein in culture supernatants was measured using commercially available ELISA kits (eBioscience, San Diego, CA, USA). ELISA was performed according to the manufacturer's instructions. All samples were assayed in duplicate.

Statistical analyses

Results were expressed as mean ± standard deviation. Data were analyzed using two-way ANOVA. Bonferroni *post hoc* tests were used to compare differences between means when significant main effects were detected. SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for all analyses, and the significance level was set at $p < 0.05$.

Results

Glucose deprivation induces IL-6 expression and release

GD enhanced IL-6 expression in C2C12 myocytes compared with GC. IL-6 expression increased significantly at 18 hours ($p < 0.01$) and 24 hours ($p < 0.05$) under GD conditions relative to GC (Figure 1A). IL-6 protein levels in the medium in both GC and GD treatments increased gradually from 0 to 24 hours. The GD treatment enhanced protein release significantly compared with the GC treatment, but IL-6 protein levels also increased under GD treatment at all of the time points ($p < 0.05$; Figure 1B).

Glucose deprivation-induced IL-6 release is mediated via ROS signaling and the p38MAPK pathway

As shown in Figure 2A, IL-6 protein level in the GC + NAC treatment was significantly lower ($p < 0.01$) than in the GC treatment. IL-6 protein level was also significantly lower ($p < 0.01$) in the GD + NAC treatment compared with the GD treatment. Tests of between-subjects effects showed that glucose deprivation and NAC interacted significantly ($F = 30.125$, $p = 0.001$), indicating that NAC almost completely inhibited IL-6 protein release under control conditions (normal culture), and glucose-deprivation induced IL-6 protein release. As shown in Figure 2B, IL-6 protein level in the GC + SB203580 treatment was significantly lower ($p < 0.01$) than in the GC treatment. IL-6 protein level was also significantly lower ($p < 0.01$) in the GD + SB203580 treatment compared with the GD treatment. Tests of between-subjects effects showed that glucose deprivation and SB203580 interacted significantly ($F = 6.695$, $p = 0.034$), indicating that SB203580 inhibited IL-6 protein release under control conditions (normal culture), and glucose deprivation-induced IL-6 protein release to a lesser extent.

Discussion

Skeletal muscle is the largest organ in the human body and produces cytokines (also called "myokines"), including IL-6, which assert influences over other tissues as well as skeletal muscle (Pedersen & Febbraio 2008).

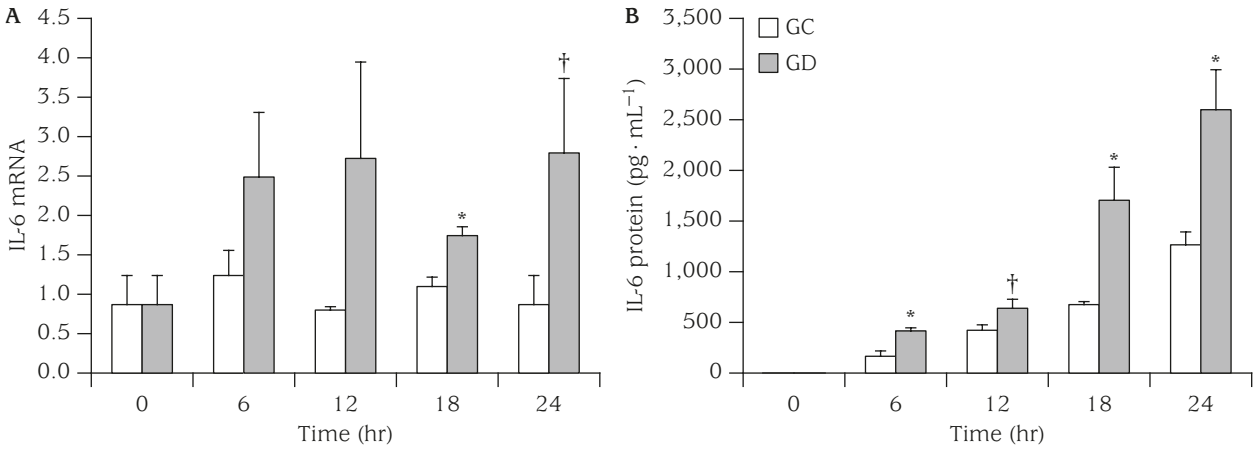

Fig. 1 Glucose deprivation induces IL-6 expression and release in C2C12 myocytes. Differentiated C2C12 myotubes were placed in DMEM medium containing 4.5 g · L^{-1} glucose (glucose control, GC) or DMEM medium without glucose (glucose deprivation, GD) for 0, 6, 12, 18 or 24 hours. (A) IL-6 mRNA levels were analyzed using RT–PCR. (B) IL-6 protein levels in media were analyzed using ELISA. Data are expressed as mean ± standard deviation. *$p < 0.01$ and †$p < 0.05$ (GD *vs.* GC at respective time points).

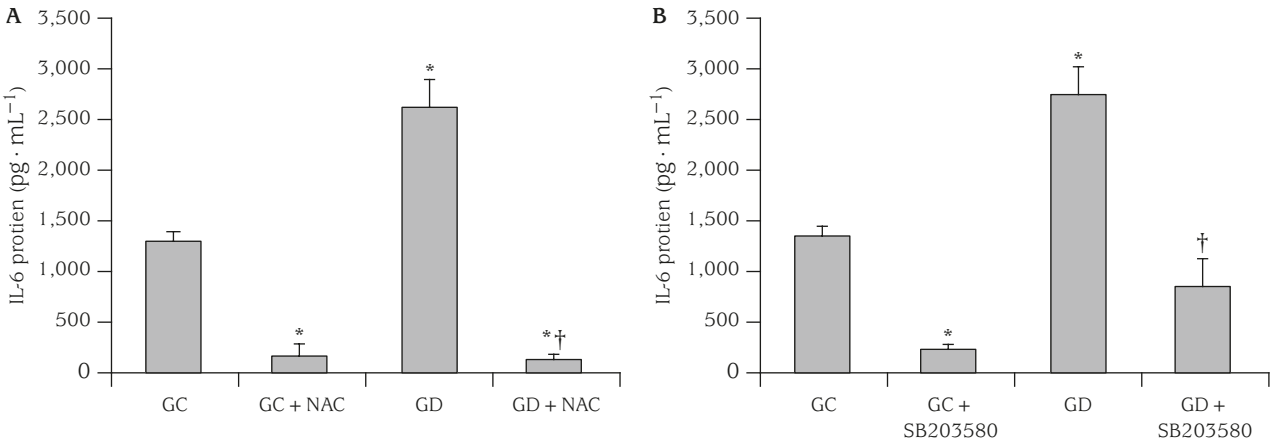

Fig. 2 Glucose deprivation-induced IL-6 release in C2C12 myocytes is mediated via ROS signaling and the P38MAPK pathway. C2C12 myotubes were incubated with either 10 mM NAC (ROS scavenger) or 10 μM SB203580 (p38MAPK inhibitor) or alone (negative control) for 30 minutes. The myotubes were then placed in DMEM media containing 4.5 g · L^{-1} glucose (glucose control, GC) or DMEM media without glucose (glucose deprivation, GD) for 24 hours. IL-6 protein levels in the media were analyzed using ELISA. Data are expressed as mean ± standard deviation. *$p < 0.01$ *vs.* GC; †$p < 0.01$ *vs.* GD.

Previously, muscle-derived exercise-induced IL-6 release was considered a consequence of inflammation and eccentric exercise-induced muscle damage, but recent studies have shown that it is linked to metabolism, and that energy availability influences its expression during prolonged exercise (Fischer 2006; Steensberg et al. 2003; Gleeson 2000). In one of our previous studies, rats exercising on a treadmill with low pre-exercise muscle glycogen levels showed significantly higher levels of plasma IL-6 during exercise ($p < 0.01$) (Tang et al. 2007). This study showed that IL-6 plasma concentration is negatively correlated with muscle glycogen content during exercise *in vivo*. Here, to eliminate the *in vivo* effects of exercise and hormones, we used the C2C12 *in vitro* cell model to investigate the direct effects of glucose deprivation on skeletal muscle-derived IL-6 expression and release. Our findings indicate that glucose deprivation strongly enhances muscle-derived IL-6 expression and release *in vitro*. Combining our findings with those of other studies, we hypothesize that changes in the level of muscle-derived IL-6 signal that muscle energy stores are reaching critically low levels.

The precise signaling events that mediate glucose deprivation-induced IL-6, however, are not well understood. Kosmidou et al. (2002) stated that ROS could stimulate IL-6 production in skeletal muscle myotubes, while Fischer et al. (2004) found that supplementation with vitamins C and E (ROS scavengers) inhibits the release of IL-6 from contracting human skeletal muscle. These studies suggest that ROS, which are primarily derived from mitochondria and crucial for redox signaling and other cellular events (Rhee 1999), are able to stimulate IL-6 production in skeletal cells. We predict that glucose deprivation results in a change in energy metabolism within mitochondria, affecting ROS production, and therefore IL-6 expression. Consistent with Kosmidou et al.'s findings, our results show that the ROS scavenger NAC almost completely inhibits both IL-6 protein release in normal cultures and glucose deprivation-induced IL-6 protein release. The p38MAPK pathway is also an important signaling pathway for regulating IL-6 expression. Chae et al. (2001) showed that blockading the p38MAPK pathway inhibits inducible nitric oxide synthase and IL-6 expression in MC3T3E-1 osteoblasts. Studies have shown that the p38MAPK pathway may be involved in ROS-mediated IL-6 production (Sano et al. 2001). Chan et al. (2004) reported that reduced carbohydrate intake resulting in low muscle glycogen leads to phosphorylation of p38MAPK in the nucleus, which appears to be an upstream target for IL-6. Here, we found that the p38MAPK inhibitor SB203580 inhibited IL-6 protein release under normal culture conditions, and also inhibited IL-6 protein release to a lesser extent under glucose deprivation conditions. Our findings clearly indicate that the p38MAPK pathway is involved in glucose deprivation-induced IL-6 protein release in C2C12 myocytes.

Taking a broader view of our result in the context of other reports, we think glucose deprivation could alter the energy metabolism of mitochondria as a result of low muscle glucose, which may then directly or indirectly affect muscle-derived IL-6 expression via ROS

signaling and the p38MAPK pathway. As a result, muscle-derived IL-6 is released into the circulatory system, where it may play a signaling role between contracting muscle and other tissues. The role IL-6 plays in obesity and insulin resistance remains controversial even after many years of research (Allen & Febbraio 2010). To further clarify the relationship between exercise-induced skeletal muscle-derived IL-6 and insulin resistance and obesity, we plan to conduct *in vitro* studies using electrical stimulation to mimic muscle contraction, in order to directly confirm our hypothesis that exercise-induced skeletal muscle-derived IL-6 can enhance energy metabolism and reduce insulin resistance.

Our study has shown that glucose deprivation induces IL-6 expression and release in C2C12 myocytes and that this glucose deprivation-induced IL-6 release is mainly mediated via ROS signaling. Skeletal muscle-derived IL-6 may play an important role in energy metabolism during exercise.

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