

Research Article

Changes of Myogenic Reactive Oxygen Species and Interleukin-6 in Contracting Skeletal Muscle Cells

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The aim of this study was to measure changes in myotube reactive oxygen species (ROS) and the production of interleukin (IL)-6 in electrically stimulated mouse C2C12 skeletal muscle cells. After five days of differentiation, myotubes were stimulated using an electrical stimulator set at 45 V at a frequency of 5 Hz, with a pulse width of 20 ms. Acute stimulations were performed for 45, 60, 75, 90, or 120 min in each dish. ROSs were detected in the extracted cells directly using a fluorescent probe. IL-6 mRNA expression in C2C12 myotubes and IL-6 concentration in C2C12 myotube supernatants were determined using real-time PCR and ELISA, respectively. Compared with control cells, ROS generation was significantly increased at 45 min after the onset of stimulation ($P < 0.01$) and continued to increase, reaching a maximum at 120 min. IL-6 mRNA expression and IL-6 concentration in C2C12 cells were significantly increased after 75 min ($P < 0.01$) and 120 min ($P < 0.05$) of electrical stimulation (ES) compared with the control cells. Our data show that a specific ES intensity may modulate ROS accumulation and affect IL-6 gene expression in contracting skeletal muscle cells.

1. Introduction

Interleukin (IL)-6 is a cytokine with varied biological effects, is expressed by mesenchymal, epithelial, and other cell types, and is unregulated in response to noxious stimuli, other cytokines, and growth factors. Numerous studies have demonstrated that IL-6 production is associated with muscle contraction and that the sympathoadrenal response to exercise plays only a minor role in the exercise-induced increase in plasma IL-6 [1]. Increases in the steady-state IL-6 mRNA level in skeletal muscle homogenates after long distance running suggest that skeletal muscle tissue might be a source of circulating IL-6 released during exercise [2]. Exercise does not induce an increase in plasma TNF- α level but induces a strong anti-inflammatory cytokine response with the appearance of IL-6 in the circulation being particularly upregulated and preceding that of other cytokines [3]. The IL-6 gene is inactive in resting muscles but is rapidly activated by muscle contraction. Additionally, IL-6 acts as an energy sensor, which is dependent on the glycogen content in muscle [4]. Low glycogen levels have been shown to induce IL-6 gene transcription in skeletal muscle during exercise.

More recent studies have shown that IL-6 release from muscle during exercise may be related to free radical metabolism, especially with reactive oxygen species (ROS) generation [5]. The generation of ROS is a normal occurrence during *in vivo* aerobic processes [6, 7]. Exercise is an effective stimulation method and is accompanied by an increased generation of free radicals, resulting in a measurable degree of oxidative modifications to various molecules [8, 9]. Although the increase in circulating levels of IL-6 after exercise is well documented, the cellular source and stimulus for its release remain elusive. A key limitation of these studies is that the experimental models were not physiological. Thus, *in vivo* studies are essential to define the role of IL-6 in skeletal muscle during exercise.

To examine whether skeletal muscle cells produce IL-6 and to identify possible stimuli for its release that are relevant to muscle contraction, we used differentiated C2C12 skeletal muscle cells (myotubes) as a skeletal cell model of electrical stimulation to imitate skeletal muscle contraction, as previously reported [10–12]. Previous data has shown that this pattern of stimulation can bring about a variety of functional

and structural changes in myotubes [13, 14]. It has been reported that control of myotube contraction using electrical pulse stimulation can induce large and rapid changes in the mRNA expression of genes encoding mitochondrial proteins. These mRNA increases result from a disruption in the equilibrium that exists between gene transcription and mRNA stability during nonadaptive steady-state conditions. Irrcher and Hood have shown that the contractile activity-mediated induction of transcription factor mRNA expression is highly divergent in C2C12 myotubes. This variability in transcription factor mRNA induction and turnover is likely important for the time-dependent, gene-specific transcription events that are responsible for many muscle phenotypic adaptations to contractile activity at the level of contractile proteins and mitochondrial biogenesis [15].

Thus, the purpose of this study was to investigate the changes in ROS level and the production of IL-6 generated by skeletal myocyte contraction. We hypothesized that ROS generation induced by skeletal muscle contraction may be one of the factors regulating muscle-derived IL-6 production and release.

2. Materials and Methods

2.1. Reagents and Cell Culture. C2C12 skeletal muscle cells were obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The cells were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μ g/mL streptomycin) in a humidified incubator at 37°C with 5% CO₂. When myoblast cultures reached confluence, they were switched to DMEM containing 2% heat-inactivated horse serum supplemented with antibiotics (differentiation medium) for five days, and the C2C12 skeletal muscle cells were differentiated into myotubes.

2.2. Electrical Stimulation Protocol. The design of the electrical stimulation apparatus was derived by modification of the existing method [16, 17]. After five days of differentiation, myotubes were stimulated using an electrical stimulator, (TY-C type), set at 45 V at a frequency of 5 Hz. Stimulations were performed acutely for 45, 60, 75, 90, and 120 min, separately. Cell extracts obtained from each dish were prepared immediately after stimulation.

2.3. ROS Measurements. A fluorescent probe, 2', 7'-dichlorofluorescein diacetate (DCFH-DA), was used for the assessment of intracellular ROS formation in cultured C2C12 myotubes [7]. This assay is a reliable method for the measurement of intracellular ROS such as hydrogen peroxide (H₂O₂), hydroxyl radical (OH•), and hydroperoxides (ROOH) [11, 16]. Myotubes were loaded with the probe and incubated for 20 min. After loading, the cells were washed in preparation for electrical stimulation, collected immediately, and then placed in the spectrofluorometer to determine the ROS production at the excitation and emission wavelengths

of 522 nm and 488 nm, respectively. The peak value of luminescence as a function of time was taken after subtracting the value of the blank signal (luminescence of the illuminated medium).

2.4. The IL-6 mRNA Measurements. Total RNA was isolated with TriZol and reverse transcribed with Moloney murine leukemia virus (M-MLV) (Promega, USA). RT-PCR was performed using Taq DNA polymerase (Promega, USA). The sequences for the primers were as follows: for β -actin cDNA: 5'-CGT GAA AAG ATG ACC CAG ATC A-3', 5'-CAC AGC CTG GAT GGC TAC GT-3'; IL-6 mRNA: 5'-TCC AGC CAG TTG CCT TCT TGG-3', 5'-TCT GAC AGT GCA TCA TCG CTG-3'. The conditions used were 94°C for 1 min, 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 min, repeated for a total of 39 cycles, final extension was performed at 72°C for 10 min. The products were run on a 1.5% agarose gel and visualized under a UV lamp. The point where the two visualized bands (200 bp from the amplification of the native IL-6 cDNA and 400 bp from the competitor molecule amplification) were of equal intensity was considered as the isopoint of the reaction (where equal starting concentrations of IL-6 cDNA and competitor existed in the PCR mix). The cycle number was chosen for each primer pair that maintained approximately exponential amplification with the enriched sample.

2.5. The IL-6 Concentrations Measurements. C2C12 cells were grown in monolayers and allowed to differentiate after reaching confluence. They were then treated with ES for 45, 60, 75, 90, and 120 min, separately. After ES treatment, the supernatants were collected and centrifuged for 20 min at 1000 g/min in a tabletop microcentrifuge to remove floating cells. Following centrifugation, pellets were discarded and supernatants used for ELISA in accordance with the manufacturer's instructions. C2C12 cell monolayers in the multiwell plates were lysed with 1 N NaOH. Protein amounts per well were determined by the Bradford method and were used to normalize against the values obtained for cytokine release.

2.6. Analysis. Electrophoretic bands were observed under UV transillumination and immediately photographed using an imaging system (SYNGENE, USA). Bandscan 5.0 (USA) band analysis software was used for analysis of electrophoretic bands to calculate the brightness of each PCR amplification product.

Data are expressed as mean \pm standard deviation (SD). The means were compared using a two-sided Student's *t*-test. Significant differences were determined using one-way ANOVA. Statistical analysis was conducted using SPSS13.0 (SPSS Inc.). Differences were considered significant at *P* < 0.05.

3. Results

3.1. Estimation of Cell Differentiation Fusion. The monolayer organization, as directly observed by inverted phase contrast microscopy, changes during the differentiation of myoblasts

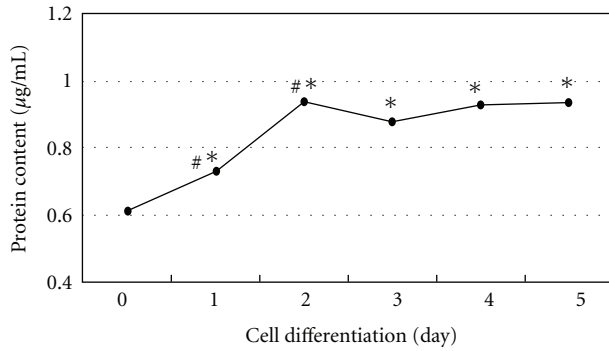


FIGURE 1: Total protein content of C2C12 myotubes during cell differentiation. When the C2C12 skeletal muscle cells were differentiated into myotubes, the absorbance of each well (OD) was measured by standard curve with a microplate reader at 450 nm wavelength, and then the protein content was calculated through the standard curve. * $P < 0.05$, significant difference compared with the first day, # $P < 0.05$, significant difference compared with the previous day.

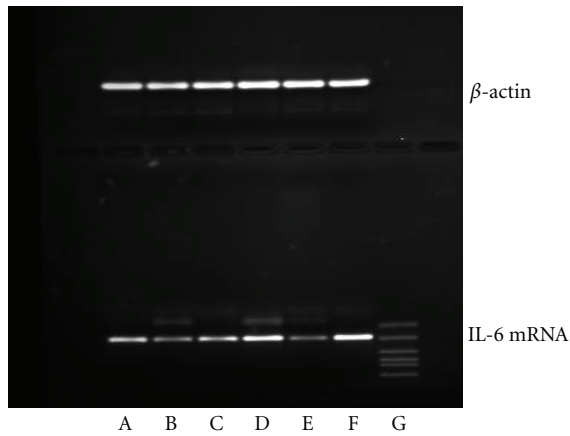


FIGURE 2: Gel photo of IL-6 mRNA expression from C2C12 myotubes following electrical stimulation. The products were run on a 1.5% agarose gel and visualized under a UV lamp. A: CN; B: ES 45 min; C: ES 60 min; D: ES 75 min; E: ES 90 min; F: ES 120 min; G: marker.

to myotubes. In the undifferentiated condition, myoblasts appear as fusiform or star-shaped cells, mostly flattened and closely adherent to the substrate. At the initial differentiation stage, intercellular spaces disappear, cells progressively align and occasionally elongate.

The bicinchoninic acid (BCA) assay was used to measure the synthesis and decomposition of C2C12 myotube intracellular protein content. Figure 1 shows the changes in total protein content, which significantly increased from the undifferentiated state to the final phase. Total protein content increased, reaching maximal values at the 3rd day of differentiation, and then steadily increased and showed minimal values in the late differentiation stage.

3.2. ROS Generation after Acute Electrical Stimulation. To ensure accurate quantification of ROS generation, we applied the fluorescent probe DCFH-DA to mark cells after ES and

TABLE 1: ROS generation during different electrical stimulation times.

	Mean \pm SD		Mean \pm SD
Control	48.94 \pm 0.2547	ES75	592.30 \pm 3.398*#
ES45	341.54 \pm 3.4034*#	ES90	401.52 \pm 4.6214*
ES60	503.52 \pm 3.3929*#	ES120	631.74 \pm 3.5515*#

The optical density (OD) values were measured using a fluorescent spectrofluorometer after ES. The peak value of luminescence as a function of time was taken after subtracting the value of the blank signal (luminescence of the illuminated medium).

* $P < 0.01$, significant difference compared with the control group,

$P < 0.05$, significant difference compared with the 90 min ES group.

TABLE 2: IL-6 mRNA expression during different electrical stimulation times.

	Mean \pm SD		Mean \pm SD
Control	0.5510 \pm 0.1637	ES75	0.7748 \pm 0.070**
ES45	0.5679 \pm 0.05984	ES90	0.5701 \pm 0.0114
ES60	0.6417 \pm 0.1280	ES120	0.7367 \pm 0.1266*

* $P < 0.05$, significant difference compared with the control group,

$P < 0.01$, significant difference compared with the control group.

subsequently measured the optical density (OD) values using a fluorescent spectrofluorometer. Table 1 shows that ROS generation significantly increased at 45 min after the onset of stimulation ($P < 0.01$) and continued to increase, reaching a maximum at 120 min, compared with control cells.

3.3. IL-6 mRNA Expression and IL-6 Concentration during Different Electrical Stimulation Times. IL-6 mRNA expression in C2C12 myotubes changed after ES (Table 2). Figure 2 shows the gel photo of IL-6 mRNA expression from C2C12 myotubes after ES. The results show that IL-6 mRNA concentration in C2C12 myotube supernatants changed with differing times of ES.

IL-6 mRNA expression in C2C12 myotubes changed following electrical stimulation. IL-6 mRNA expression in C2C12 myotubes was significantly increased after 75 min ($P < 0.01$) and 120 min ($P < 0.05$) of ES compared with the control group. Compared with the 90 min ES group, IL-6 mRNA expression in the 75 and 120 min ES groups was significantly increased ($P < 0.05$).

IL-6 protein concentration from C2C12 myotube supernatants was changed after ES. IL-6 concentration increased in C2C12 myotube supernatants with increasing ES time compared with the control group, with the peak values appearing at 75 and 120 min ($P < 0.01$). Compared with the 90 min ES group, IL-6 concentration in the 75 and 120 min ES groups was significantly increased ($P < 0.05$) (Table 3).

4. Discussion

Contractile activity is a potent stimulus for the induction of numerous cellular adaptations in skeletal muscle [18]. To assess alterations in response to contractile activity, we used an isolated cellular system in the absence of neural and

TABLE 3: IL-6 concentration during different electrical stimulation times.

	Mean \pm SD (pg/mL)		Mean \pm SD (pg/mL)
Control	81.83 \pm 13.16	ES75	2800 \pm 69.28*#
ES45	347.17 \pm 59.28*	ES90	274.17 \pm 13.58*
ES60	674.33 \pm 51.99*#	ES120	675.33 \pm 86.09*#

* $P < 0.01$, significant difference compared with the control group,

$P < 0.05$, significant difference compared with the 90 min ES group.

humoral factors. The results show that electrical stimulation (45 V, 5 Hz, 20 ms) modulates ROS accumulation and affects the gene expression level of IL-6. However, these changes did not cause any significant cell damage, implying that the intracellular redox balance was maintained. Skeletal muscle myotubes displayed a large and rapid increase in ROS production during ES. ROS release after different contraction times *in vitro* has not been previously described. However, abundant *in vivo* evidence has demonstrated that ROS production is a stimulus for skeletal muscle adaptation to exercise training [8, 19]. Previous reports support the concept that exercise-induced ROS generation alters muscle gene expression and contributes to exercise-induced adaptations of skeletal muscle *in vivo* [20]. A common approach in many of these studies is to abolish the signaling effects of exercise-induced ROS production in skeletal muscle by treating animals with antioxidants. For example, two independent studies have recently concluded that antioxidant supplementation can impede important training adaptations in human skeletal muscle [21, 22]. Our previous studies have also demonstrated that ES of C2C12 myotubes can induce rapid increases in ROS, induce cellular dysfunction, and induce mitochondrial dysfunction [11, 21]. This change is inconsistent with results previously reported *in vivo* [8, 20].

ROS production in muscle cells elicited by ES is related to the voltage, frequency, and duration applied. Different intensities of ES can induce different changes in ROS production [7]. Silveira et al. used different ES intensities on muscle cells and found that intense ES (50 V, pulse width 1 ms, pulse interval 0.01 s) of muscle cells increased intra- and extracellular DCF fluorescence, a measure of oxidation, by 171% and 105%, respectively, compared with control nonstimulated cells. In contrast, moderate stimulation (50 V, pulse width 1 ms, pulse interval 0.1 s) did not significantly increase the extracellular DCF fluorescence [7]. Increasing the stimulation frequency increases skeletal muscle contraction strength, which leads to increases in skeletal muscle mitochondrial activity and superoxide generation. McArdle et al. used ES (30 V, 1 Hz, pulse width 2 ms) on differentiated rat satellite cells to study the time course of ROS release. The authors found that a short (15 min) contraction in satellite cells caused a rapid 40x increase in ROS [23]. This increase in ROS can cause skeletal muscle protein thiol modification, a transient decrease in SOD and catalase activity, and an increase in heat shock protein concentration. However, these changes did not cause any notable cell damage [24].

In this study, we used ES (45 V, 5 Hz) to simulate C2C12 myotubes. We found that ROS production significantly

increased with ES compared with the control nonstimulated cells, and the change in ROS generation followed a triphasic curve pattern, with the peaks appearing in the 75 and 120 min ES groups. This is similar to our previous findings using the same mode of ES [11, 12]. Our previous results showed that exercise-induced ROS changes are related to mitochondrial oxygen consumption and the regulation of the antioxidant system. Many *in vivo* studies have also shown that oxidative stress in skeletal muscle is a hallmark of pathophysiological states [19]. ROS production was evident across the physiological range of membrane potentials and was relatively insensitive to membrane potential changes [25]. Abnormally high levels of ROS and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes. We hypothesized that ROSs are removed mainly by cellular antioxidant systems, which include antioxidant vitamins, protein and nonprotein thiols, and antioxidant enzymes under physiological conditions. However, when ROS production is excessive, such as during prolonged aerobic exercise, cellular antioxidant systems may be overwhelmed, leading to extensive cell and tissue damage. The exercise-induced IL-6 production and secretion within skeletal muscle fibers suggests that IL-6 may play a role in energy supply and storage in muscle during and after exercise. The role is likely short term, physiological, and locally active within minutes or a few hours [26]. In the present study, we observed the changes in IL-6 mRNA expression and IL-6 protein content in C2C12 myotubes at different ES times (0, 45, 60, 75, 90, and 120 min). This is different from previous studies, which suggest that the IL-6 mRNA level in skeletal muscle is markedly increased during exercise [27–29]. IL-6 protein has been shown to accumulate in contracting muscle fibers and be released in large amounts into plasma. Furthermore, transcriptional activation of the IL-6 gene in the skeletal muscle of humans is highly elevated during prolonged exercise [5, 30]. Additional studies have shown that the time and intensity of exercise required to accumulate IL-6 protein within contracting muscle are not well defined. However, duration of exercise has been shown to be the single most important factor that determines the magnitude of the systemic IL-6 response [29]. Our results suggest that IL-6 mRNA expression in C2C12 myotubes changed with differing ES times. IL-6 mRNA expression in both C2C12 myotubes and supernatants was significantly increased in the 75 and 120 min ES groups, compared with the control group.

Few studies have addressed the relationship between myogenic ROS and IL-6 after exercise *in vivo*. Some studies have shown that supplementation with vitamins C and E attenuated the systemic IL-6 response to exercise in plasma and skeletal muscle [28, 31]. Antioxidants, such as vitamins C and E, have been shown to attenuate oxidative stress at rest as well as in response to exercise, thereby reducing the generation of ROS [32]. Using C2C12 myotubes incubated with H₂O₂ at different concentrations, Kosmidou et al. found that IL-6 content of cultured supernatants increased dependently after 24 h. The authors concluded that ROSs stimulate IL-6 production from skeletal myotubes by increasing transcriptional activation of the IL-6 gene through an NF- κ B-dependent pathway [33]. In human bronchial epithelial cells,

ROS-stimulated IL-6 production is preceded by increases in IL-6 steady-state mRNA levels [34]. Keller et al. have suggested that induction of IL-6 expression in contracting myofibers may be largely responsible for the rise in circulating IL-6 concentration during prolonged exercise [30]. Therefore, we speculate that similar mechanisms are likely active in contracting skeletal muscle and that ROS generation may play a role in the signaling processes by which C2C12 myotubes adapt to changes in the pattern or duration of contractile activity.

It is well known that a variety of stresses can induce IL-6 upregulation. However, studies on the IL-6 response to electrical stimulation are scarce. To the best of our knowledge, this is the first report addressing the IL-6 response to ES. In the present study, we have shown that ES can cause changes in IL-6 mRNA expression, IL-6 protein content, and variable increases in ROS generation. We speculate that myogenic ROS generation is likely to mediate myogenic IL-6 generation. However, in this study, we only investigated the possible link between myogenic IL-6 and ROS after ES, not ROS produced by muscle vasoconstriction and subsequent IL-6 release from skeletal myotubes. Thus, we cannot exclude the possibility that other intramuscular metabolic changes induce myogenic ROS production and subsequent release of IL-6 during exercise. Further studies are needed to investigate this possibility.

Our results indicate that electrical stimulation (45 V, 5 Hz, 20 ms) may modulate ROS accumulation and affect the gene expression level of IL-6. Furthermore, the variation trend is consistent with the stimulation time. We speculate that ROS generated by skeletal muscle cell contraction may be one of the factors regulating muscle-derived IL-6 production and release.

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