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Differential regulation of IL-6 and TNF-α via calcineurin in human skeletal muscle cells

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Abstract

Interleukin-6 increases in skeletal muscle during exercise, and evidence points to Ca^{2+} as an initiator of IL-6 production. However, the signalling pathway whereby this occurs is unknown. One candidate for Ca^{2+} -mediated IL-6 induction is calcineurin, an activator of NF-AT. Here we investigated whether skeletal myocytes produce IL-6 in a Ca^{2+}/calcineurin-dependent manner, and whether TNF-α, an inducer of IL-6, is affected by these stimuli. Human skeletal muscle cell cultures were stimulated with ionomycin time-and dose-dependently to elevate intracellular Ca^{2+} levels, with or without addition of cyclosporin A (CSA); a calcineurin inhibitor. mRNA was extracted from myocytes and analysed for IL-6 and TNF-α gene expression. IL-6 mRNA increased time- and dose-dependently with ionomycin stimulation, an effect that was blunted by ~75% in the presence of CSA. In contrast, TNF-α gene expression was decreased by ~70% in response to ionomycin treatment, but increased in response to addition of CSA. These data demonstrate that IL-6 and TNF-α are regulated differentially in skeletal muscle cells in response to a Ca^{2+} stimulus. Blocking the calcineurin pathway resulted in inhibition of the IL-6 response to ionomycin, whereas TNF-α increased by addition of CSA, further indicating a differential regulation of IL-6 and TNF-α in human skeletal myocytes.

Keywords: IL-6; TNF-α; Ca^{2+}; Calcineurin; Cyclosporin A

1. Introduction

Studies from the previous 10 years indicate that the interleukin-6 (IL-6) response to exercise may have an important metabolic role. Plasma IL-6 increases time- and dose-dependently in response to exercise [1–3], where skeletal muscle cells are major contributors of IL-6, as both in situ hybridisation and immuno-histochemistry analysis of muscle biopsies have shown the presence of both IL-6 mRNA and protein, respectively [4,5]. Stimulation of skeletal muscle cells with inflammatory substances such as lipopolysaccharide (LPS), or the pro-inflammatory cytokines IFN-γ and TNF-α results in a release of IL-6, showing that myocytes are able to produce IL-6 also in response to inflammatory stimuli [6,7]. Recent findings have identified a hormonal role of IL-6 released from skeletal muscle during exercise. Thus, contracting muscle fibres produce and release IL-6 in an endocrine manner to facilitate substrate mobilisation from liver and adipose tissue. In addition, IL-6 enhances lipid oxidation possibly via activation of AMPK [8].

Several pathways may regulate myocyte IL-6 production during contraction. However, the release of Ca^{2+} from the sarcoplasmatic reticulum (SR) at the onset of contraction seems to be one major initiator of this production, and elevated levels of Ca^{2+} in myocytes and rat muscle result in an enhanced IL-6 expression [9,10]. p38MAPK seems to

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play a role in Ca^{2+} dependent IL-6 production [9]. However, other Ca^{2+} dependent signalling pathways may also affect IL-6 production in skeletal muscle. In particular, calcineurin has gained interest in the field of cytokine signalling, as it activates the Nuclear Factor of Activated T-cells (NF-AT) by dephosphorylation, leading to nuclear translocation and increased transcription of several cytokines [11], including IL-6 in vascular smooth muscle cells [12]. Activation of calcineurin in skeletal muscle has been demonstrated when primary skeletal muscle cell cultures are stimulated electrically [13], and in response to exercise in humans [14]. Thus, this signalling pathway may constitute induction of IL-6 in response to muscle contractions.

In contrast to IL-6, the pro-inflammatory cytokine TNF-α only shows a trend to increase in skeletal muscle in response to exercise, and TNF-α is not released from contracting skeletal muscle to the circulation [15]. Consequently, the production of IL-6 in response to exercise is not likely to be induced by TNF-α. Though much attention has been drawn to the regulation of IL-6 in skeletal muscle, not much is known about the expression of TNF-α in response to exercise in this tissue. IL-6 is increased in skeletal muscle by elevating Ca^{2+} levels, but the effect of such stimulus on TNF-α is unknown. TNF-α is regulated by changes in Ca^{2+}/calcineurin within lymphocytes [16], however, whether this is also seen in myocytes is not known, and since TNF-α levels within skeletal muscles are not closely related to contraction it may not be the case. Theoretically, there may even be an inverse relationship, as high levels of IL-6, as seen during exercise, seem to blunt TNF-α [17].

In the present study, we aimed to investigate whether skeletal myocytes produce IL-6 in a Ca^{2+}/calcineurin dependent manner, and whether this response would increase time- and dose-dependently in a similar fashion as the IL-6 production is increased with exercise duration and intensity in skeletal muscle. In addition, we aimed to investigate whether Ca^{2+} also induces TNF-α formation in skeletal myotubes, as these two cytokines are often linked to the same events in inflammation, obesity, and type II diabetes [18–20]. Furthermore, we hypothesised that addition of cyclosporin A (CSA), a calcineurin phosphatase inhibitor [11], would blunt the Ca^{2+}-induced expression of IL-6. IL-6 and TNF-α responses were evaluated on the gene expression level, as previous studies have demonstrated that these cytokines are strongly regulated at the level of gene transcription [21,22].

2. Methods

2.1. Promocell muscle cells

Commercially available human muscle cells (Cat # C-10531, Promocell, Germany) were used in the study. Cells were seeded and grown according to the manufacturers directions using skeletal muscle growth medium from Promocell. Cells proliferated for 2–3 passages and were subsequently differentiated on matrigel (Becton–Dickinson) for 7–10 days, resulting in multinucleated, myotube structures.

Human primary myoblasts differentiate and fuse into myotubes after approximately 7–10 days in culture. Investigations of myosin heavy chains of myotubes show that they are primarily embryonic and neonatal [23]. Nevertheless, examination of many different proteins and pathways in cultured muscle cells suggest that, although some differences do exist, these cells contain much of the same proteins and signalling systems as mature muscle [24–26]. Therefore, this model of primary human skeletal muscle cells is a useful and physiological relevant model to use for the study of cellular mechanisms in human tissue, such as the regulation of IL-6 gene transcription that cannot easily be studied in human subjects.

2.2. Drug treatment

2.2.1. Ionomycin

For dose–response curves, cells were incubated for 12 h in 0.5 nM, 1 nM, or 2 nM of ionomycin, and for a time–response, cells were incubated with 1 nM ionomycin for 3 h, 6 h, 9 h, and 12 h in serum-free medium. Control cell samples were incubated for 12 h without ionomycin. At the end of incubation, cells were scraped off the dish and transferred in 200 μl of PBS to an Eppendorf tube and quickly frozen in liquid nitrogen.

2.2.2. Cyclosporin A

Two different doses of CSA (2.5 mM or 5 mM) were added to the cell culture medium, in combination with 1 nM ionomycin for 12 h of incubation. Control samples were incubated for 12 h without ionomycin and CSA. For CSA time–response curves, cells were incubated with a fixed dose of ionomycin (1 nM) and CSA (5 mM) for either 6 h or 12 h. At the end of incubation, the cells were scraped off the dish and transferred in 200 μl of PBS to an Eppendorf tube and quickly frozen in liquid nitrogen.

2.3. RNA extraction

RNA extraction was performed in accordance with the TRIzol distributor directions (Life Technologies, cat # 15596-026) with a few modifications.

Cells were lysed in 1 ml of TRIzol reagent by repetitive pipetting followed by incubation of the homogenised sample for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Subsequently, 0.2 ml of chloroform was added, followed by vigorous shaking for 15 s and incubation at room temperature for 2–3 min. The samples were centrifuged at 12,000*g* for 15 min at 4 °C. The upper aqueous phase was transferred to a fresh tube and was mixed with 0.5 ml of isopropanol, and incubated at room temperature for 10 min followed by centrifugation at 12,000*g* for 10 min at 4 °C. The supernatant was removed by inversion and the pellet was washed