Ionomycin, But Not Physiologic Doses of Epinephrine, Stimulates Skeletal Muscle Interleukin-6 mRNA Expression and Protein Release

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It has been hypothesized that epinephrine may stimulate interleukin (IL)-6 gene expression in skeletal muscle. The aim of the present study was to examine the effect of epinephrine on IL-6 gene expression within, and protein release from, skeletal muscle. We hypothesized that physiologic epinephrine would neither result in an increase in IL-6 mRNA nor protein release from skeletal muscle. Soleus muscle was excised from 4-week-old anesthetized Sprague Dawley rats and incubated in a Krebs buffer with the addition of either saline (CON), epinephrine, at concentrations of 1,000 nmol/L (EPI 1,000), 100 nmol/L (EPI 100), or 10 nmol/L (EPI 10), or the calcium ionophore, ionomycin (IONO), a positive control. After a 1-hour incubation, muscle was collected and extracted for RNA, reverse transcribed, and IL-6 gene expression was determined by real-time polymerase chain reaction (PCR). An aliquot of incubation medium was also collected and analyzed for IL-6 protein by enzyme-linked immunosorbent (ELISA). EPI 1,000 and IONO increased ($P < .05$) IL-6 mRNA, whereas EPI 100 and EPI 10 were without effect. IL-6 protein release from skeletal muscle was increased in IONO ($P < .05$), but not in CON or EPI at any concentration. These data demonstrate that while pharmacologic concentrations of epinephrine activate IL-6 mRNA, supraphysiologic and high-physiologic doses appear to have little, if any, effect on IL-6 gene transcription in skeletal muscle. In addition, ionomycin can stimulate IL-6 gene expression and protein release after only 1 hour of exposure.

INTERLEUKIN-6 (IL-6) is a multifunctional immune-modulating cytokine that is elevated in stressed states and in patient populations. Expression of IL-6 is putatively associated with insulin resistance, visceral obesity, atherosclerosis, and glucocorticoid receptor dysfunction (for review see Febbraio et al[1]). It is not surprising that IL-6 is associated with obesity-related disorders as it is released from adipose tissue[2] and positively correlates with body fat levels.[3,4] Recent evidence suggests, however, that IL-6 is produced by contracting skeletal muscle,[1,5] but the precise mechanism(s) that regulate IL-6 production by skeletal muscle are unclear. Of note, skeletal muscle IL-6 mRNA expression is not elevated in insulin-resistant humans, nor is it activated by insulin in these patients,[4] suggesting that factors associated with physical exercise and/or muscle contraction regulate IL-6 transcription in muscle.[6]

It is well known that during exercise the increase in plasma epinephrine is marked, and it has often been hypothesized that the increase in circulating epinephrine could mediate, in part, the IL-6 response.[7-9] Indeed, there is evidence to suggest that adrenergic regulation may be important in regulating IL-6. Administration of epinephrine increases plasma IL-6 in both rats[10] and humans.[11] Furthermore, plasma epinephrine concentrations correlate with plasma IL-6,[12] while the release of IL-6 from skeletal muscle has been shown to be positively correlated to exercise intensity and arterial epinephrine concentration[13] during exercise in humans. However, these studies do not provide evidence that epinephrine increases IL-6 production in skeletal muscle, because they either did not identify the source of the systemic increase when either animals or humans were infused at rest, or the present associative data collected during exercise.

In contrast, during exercise, plasma IL-6 increased 30-fold, however, an epinephrine infusion mimicking closely the exercise-induced increase in plasma epinephrine increased plasma IL-6 only 4-fold,[14] suggesting that during exercise the role of plasma epinephrine on the IL-6 response is relatively minor. In addition, during 2 legged knee extensor exercise, both the IL-6 gene transcription and protein release was augmented in a leg previously depleted of glycogen, even though both legs were subjected to the same level of circulating epinephrine,[15] also suggesting that factors other than epinephrine play a more important role in IL-6 production during contraction. The direct effect of epinephrine on IL-6 gene expression and protein release from skeletal muscle has not been experimentally investigated, and this was the primary aim of the present study. Using an in vitro rat skeletal muscle incubation model, we hypothesized that physiologic epinephrine would neither result in an increase in IL-6 mRNA nor protein release from these muscles.

The rapid increase in IL-6 nuclear transcription rate seen within the first 30 minutes of exercise has been proposed to be attributable to cytosolic calcium (Ca^{2+}) levels.[16] The role of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the control of a diverse range of cellular functions, including gene expression and proliferation, is well documented[17,18] and the $[Ca^{2+}]_i$ concentration has been implicated in signalling cascades as a potent signalling factor for IL-6 transcription.[1,6] Indeed, it has been demonstrated that the calcium ionophore, ionomycin, can increase IL-6 gene expression in cultured human primary muscle cells after a 6-hour incubation period, and further so by 48 hours.[19] The effect of Ca^{2+} stimulation on IL-6 expression has only been investigated under such long-term circumstances, and whether this response has a more rapid effect, or whether ionomycin

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treatment results in IL-6 protein release has not been investigated. This was the secondary aim of this study, and we hypothesized that ionomycin administration would result in IL-6 gene transcription in, and protein release from, skeletal muscle.

MATERIALS AND METHODS

Animals and Muscle Preparation

Male Sprague-Dawley rats of 4 weeks of age and 85 ± 3 g body weight were purchased from Monash Medical Centre (Clayton, Victoria, Australia). The animals were housed in the RMIT University animal facility in a controlled environment with a 12:12 hour light:dark cycle and fed rat chow and water ad libitum. All experimental procedures were approved by the Animal Ethics Committee of RMIT University. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg · 100 g^{-1} body weight, Nembutal; Rhone Merieux, Queensland, Australia), and the soleus muscle from both hind limbs were excised from tendon to tendon. Care was taken to avoid damage to the muscle during surgery, and animals were immediately euthanized with an overdose of Nembutal.

Muscle Metabolite Experiments

Viability of our soleus muscle preparation was established in a preliminary experiment. Paired muscles were isolated as described. To determine the viability of the muscle over the incubation period, 1 muscle was rapidly frozen, whereas the contralateral muscle was incubated for 60 minutes in gassed Krebs buffer (see below for details), then snap-frozen in liquid nitrogen. Muscles were freeze-dried and powdered, and nonmuscle contaminants were removed. The freeze-dried muscle was extracted in 0.5 mol/L $HClO_4$ (1 mmol/L EDTA) and neutralized with 2.2 mol/L $KHCO_3$. Adenosine triphosphate (ATP), phosphocreatine (PCr), creatine, and lactate were subsequently determined by spectrophotometric assays.[20,21]

Experimental Protocol

Each muscle was preincubated in a 20-mL vial containing 3 mL pregassed (95 % O_2, 5% CO_2) Krebs buffer (8 mmol/L glucose) at 30°C. After preincubation, either Krebs buffer (CON, n = 5), 10 μmol/L ionomycin (IONO, n = 5), or epinephrine at concentrations of 1,000 (EPI 1,000, n = 8), 100 (EPI 100, n = 7), and 10 nmol/L (EPI 10, n = 9) was added to the appropriate vial and incubated for a further 40 minutes. All epinephrine stock solutions were prepared in Krebs buffer with 2-mg/mL ascorbic acid to prevent oxidation. A 3.0-mmol/L stock solution of epinephrine bitartrate (AstraZeneca, North Ryde, NSW, Australia) was diluted to 3 μmol/L daily such that the appropriate volume could be added to the bath to achieve concentrations of 10, 100, or 1,000 nmol/L EPI in a final bath volume of 3.0 mL. The total volume added for all conditions was 100 μL. At the end of the incubation, muscles were snap-frozen in liquid nitrogen and stored at −80°C for later analysis. A 1.0-mL aliquot of incubation media was also removed and stored at −80°C for later analysis.

Determination of IL-6 Gene Expression and Protein Release

The soleus muscle was extracted for total RNA followed by determination of IL-6 gene expression by real-time polymerase chain reaction (PCR). Methods and quantification methods are described elsewhere.[22] The primers and probe were designed from the rat IL-6 mRNA sequence (GenBank/EMBL Accession No. M26744). An 81-bp IL-6 gene fragment was amplified using the forward primer 5′-ATATGTTCTCAGGGAGATCTTGGAA-3′ and reverse primer 5′-GTGCATCATCGCTGTTCATACA-3′ (Sigma Geno-sys, Castle Hill, NSW, Austraila). The IL-6 protein released from the muscle into the incubation media was quantified by enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Minneapolis, MN).

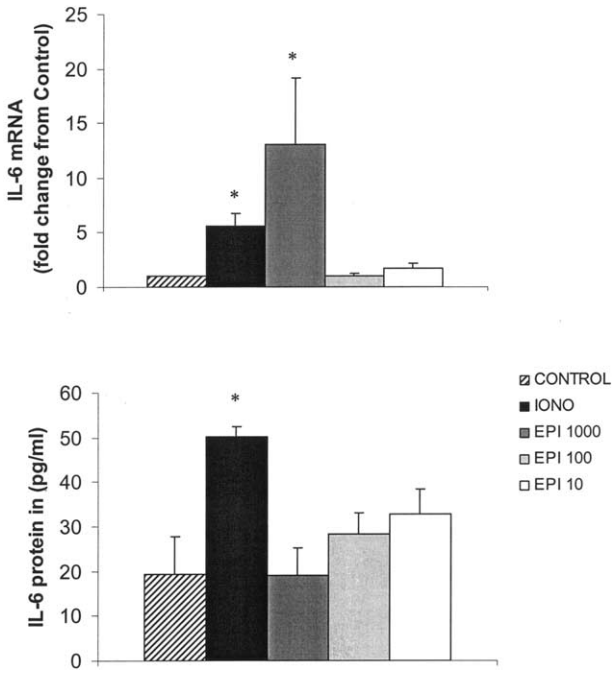

Fig 1. (A) IL-6 mRNA expression in and (B) release from rat soleus muscle incubated for 60 minutes in saline (control, n = 5), 10 μmol/L ionomycin (IONO, n = 5), 1,000 nmol/L epinephrine (EPI 1,000, n = 8), 100 nmol/L epinephrine (EPI 100, n = 7), 10 nmol/L epinephrine (EPI 10, n = 9). *Indicates difference ($P < .05$) from control. Data expressed as mean ± SEM.

Statistical Analysis

Comparisons between means were made using the paired *t* test. A *P* value of less than .05 defined statistical significance. All values are presented as mean ± SEM.

RESULTS

Isolated muscles were viable over the 60-minute incubation period as indicated by the maintenance of ATP and total creatine within the muscle (rapid freeze: 15.9 ± 0.7 and 82.2 ± 5.6; postincubation: 18.4 ± 3.0 and 90.6 ± 3.9 mmol · kg^{-1} dm, respectively). IL-6 mRNA was increased by IONO and pharmacologic levels of EPI (1,000 nmol/L; $P < .05$) compared with CON. Lower levels of EPI (10 and 100 nmol/L) were without effect on IL-6 mRNA (Fig 1, top). IL-6 protein increased ($P < .05$) in the incubation media with IONO compared with CON. Despite the observed increase in IL-6 mRNA with pharmacologic EPI, there was no measurable increase in IL-6 protein in the incubation media with EPI at all concentrations (Fig 1, bottom).

DISCUSSION

This is the first study to investigate the direct effect of epinephrine on IL-6 gene expression within, and protein release from, skeletal muscle. In the present study, IL-6 mRNA was not altered by short-term exposure to high physiologic and

supraphysiologic epinephrine concentrations, whereas epinephrine, at a concentration of more than 1,000 times that occurring physiologically, while increasing IL-6 mRNA, did not increase IL-6 protein release. These data indicate that epinephrine is unlikely to be the stimulus for the exercise-induced increase in IL-6 gene expression and does not increase IL-6 protein release from skeletal muscle.

Helge et al[13] correlated plasma IL-6, released from skeletal muscle during exercise, with arterial epinephrine concentrations, and Nieman et al[9] suggested that during a prolonged exercise bout differences in IL-6 gene expression were due to differences in blood epinephrine levels. Previous studies have investigated the effect of epinephrine infusion on IL-6 levels. DeRijk et al[10] infused epinephrine to physiologic concentrations and observed an increase in plasma IL-6, and in the face of a 24-fold increase in epinephrine concentration Søndergaard et al[11] saw a 2-fold to 3-fold increase in plasma IL-6. These studies have reported measurements of circulating factors and have not isolated the impact of epinephrine on skeletal muscle IL-6 per se. Based on our present data it appears, therefore, that in the studies mentioned above, the source of the IL-6 was not the skeletal muscle. It must be noted, however, that in the present study we investigated the effects of epinephrine per se on the IL-6 response in muscle and did not excise muscle from animals following contraction. Thus, we cannot rule out the possibility that following exercise the relationship between epinephrine and IL-6 may differ, because the high energy phosphagen content of contracting compared with noncontracting muscle may differ markedly, and this may affect the response to epinephrine.

It is well known that intracellular calcium ($[Ca^{2+}]_i$) controls a diverse range of cellular functions including gene expression and proliferation, and the literature suggests that $[Ca^{2+}]_i$ is a potent signalling factor for IL-6 transcription.[19] Our finding that the Ca^{2+} ionophore, ionomycin, increased the IL-6 gene expression is consistent with previous findings.[19] Keller et al[19] incubated isolated human muscle cells with ionomycin and reported progressive increases in IL-6 mRNA from 6 to 48 hours of incubation. In the present study, we extended on their findings and demonstrated increased IL-6 gene expression within 1 hour. Moreover, these data demonstrate that Ca^{2+} increases IL-6 protein release from skeletal muscle. The present finding showing that the Ca^{2+} ionophore ionomycin increases IL-6 protein release from skeletal muscle is consistent with marked increases in IL-6 protein release seen within 30 minutes of exercise,[23] which coincides with a period when there is a rapid increase in cytosolic Ca^{2+} levels due to mechanical load.[24] Ca^{2+} signals are known to propagate to the nucleus where they activate or inhibit the function of various transcription factors, and hence, influence gene transcription. Some of the signals downstream of Ca^{2+} have been identified as activators of cytokine gene transcription,[1] but the degree to which IL-6 is activated in skeletal muscle by such signalling pathways is not known.

It is noteworthy, that while IONO increased both IL-6 mRNA and protein release, EPI 1,000, did not result in protein release (Fig 1B), despite the fact that the mRNA increase was marked (Fig 1A). It is difficult to interpret these results, however, the dose of epinephrine in these circumstances was pharmacologic, and the stimulus for IL-6 mRNA transcription may have been different compared with IONO, resulting in post-transcriptional modification. Alternatively, the release of IL-6 may have been impaired under these circumstances.

In conclusion, we have demonstrated for the first time that calcium stimulates IL-6 gene expression within, and protein release from skeletal muscle, but that physiologic concentrations of epinephrine do not stimulate IL-6 gene expression within or protein release from rat soles muscle in vitro.

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