IL-6 and TNF-α expression in, and release from, contracting human skeletal muscle

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In addition to the large body of research examining the immunological effects of elevations in TNF-α and IL-6, recent studies have also focused on the metabolic effects of these cytokines. Both have been found to be expressed in human skeletal muscle (29, 31), and both are associated with insulin resistance and type 2 diabetes (15). Work from our laboratory has focused on the origin of the exercise-induced increase in IL-6. We have demonstrated that muscle contraction rapidly increases IL-6 gene expression in skeletal muscles in both rats (12) and humans (21, 31, 33). In addition, we have shown that the intramuscular nuclear transcriptional activity of IL-6 is rapidly increased with the onset of exercise (14) and that IL-6 protein is released from skeletal muscle during prolonged exercise (33, 34). The contraction-induced increased transcriptional activity of the IL-6 gene and the IL-6 protein release is further elevated when intramuscular glycogen stores are low (14, 33). We have also recently shown that cultured human primary muscle cells are capable of increasing IL-6 mRNA when incubated with the calcium ionophore ionomycin (13). Therefore, it is likely that myocytes produce IL-6 in response to muscle contraction, an effect that is exacerbated by low intramuscular glycogen stores, and production of IL-6 by such tissue accounts for the exercise-induced increase in plasma levels of this cytokine.

In contrast with the well described literature concerning IL-6, less is known regarding the cells/organisms responsible for the increased plasma levels of TNF-α observed during strenuous exercise (21, 32). TNF-α is expressed in human skeletal muscle (29), and it is therefore possible that contracting skeletal muscle is the source of this increase. However, TNF-α expression is augmented in the skeletal muscle of patients with type 2 diabetes (29), and it decreases insulin-stimulated rates of glucose storage in cultured human muscle cells (11). Furthermore, TNF-α administration impairs both insulin-mediated capillary recruitment and glucose uptake in anesthetized rats (42), whereas TNF-α-null mice are protected from insulin resistance (39).

Cytokines are a group of proteins produced by many cells and/or tissues in response to stress (1). Given that physical exercise can markedly disrupt cellular homeostasis, it is not surprising that exercise elevates plasma levels of several cytokines, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α (22). Until recently, it was hypothesized that the exercise-induced increase in these cytokines was a consequence of an immune response due to local damage in the working muscles (19). However, work from our research group has demonstrated that the immune cells are not the source of the increase in plasma TNF-α or IL-6 during exercise (32).

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It is well known that muscle contraction rapidly increases glucose uptake; therefore, an exercise-induced increase in TNF-α would, on the one hand, appear paradoxical. On the other hand, cytokines are produced in many cells and/or tissues in response to stress, and given that muscle contraction can markedly disrupt muscle cell homeostasis, it is possible that such cells may be producing TNF-α during contraction. To our knowledge, no studies have examined the TNF-α expression in contracting skeletal muscle or whether TNF-α is released from muscle during contraction.

Although we have presented hypotheses as to the biological role of IL-6 expression in contracting muscle (23), we have yet to determine the precise biological role. Of note, it has been demonstrated that IL-6 can impair TNF-α expression in cardiac muscle (35); therefore, one potential role of IL-6 expression in contracting muscle may be to downregulate TNF-α expression. Because the contracting muscle is the organ that undergoes the greatest disruption to homeostasis during exercise, we aimed to determine whether this organ is responsible for the observed increase not only in IL-6 but also in TNF-α during exercise. We hypothesized that the skeletal muscle will release IL-6, but not TNF-α, during exercise because of the previous observations that TNF-α promotes impaired glucose uptake in skeletal muscle. In addition, we aimed to determine whether the TNF-α expression increased during contraction and whether the kinetics of any increase differed from the expression of IL-6, so that we could explore the possibility that a role for IL-6 expression in skeletal muscle may be to downregulate any increase in TNF-α expression.

MATERIALS AND METHODS

Subjects

Six healthy, physically active but not specifically trained, male subjects [mean age 26 yr (range 22–33), mean weight 78.1 kg (range 70–93), mean height 1.87 m (range 1.75–1.93 m)] were recruited to participate in the study. The subjects were given both oral and written information about the experimental procedures before giving their written informed consent. The study was approved by the Copenhagen and Frederiksberg Ethics Committees, Denmark.

Experimental Design

Preexperimental protocol. Each subject underwent preliminary exercise tests on the two-legged knee-extensor apparatus (5). After they became familiar with the knee-extensor exercise model, they underwent a maximal exercise test to determine their individual knee-extensor peak power output (W\textsubscript{max, ke}). Thereafter, they performed ~2 h of two-legged knee-extensor exercise at ~55% W\textsubscript{max, ke} (mean workload = 93 ± 4 W) to become fully accustomed to performing the exercise for prolonged periods. At 1700 on the day before the experimental trial, subjects reported to the laboratory and performed 60 min of upright bicycling exercise, which subjects could tolerate for the duration of the exercise period. Subjects were then provided with a diet consisting of 300 ml of fruit juice, 500 ml of cola, 100 g of raisins, and 500 g of pasta (20% protein, 75% carbohydrate, 5% fat). This diet was consumed by 2100 on the evening before the experimental trial.

Experimental procedures. On the day of the experiment, subjects reported to the laboratory at 0730, voided, changed into appropriate exercise attire, and rested in a supine position. Catheters were then placed in the femoral vein and femoral artery of one limb with subjects under local anesthesia (Lidocaine, 20 mg/ml), as previously described (2). A blood sample was then drawn into a precooled tube containing EDTA, mixed, and immediately centrifuged at 2,200 g for 15 min at 4°C. The plasma was stored at −80°C until analysis. At this point, the femoral arterial blood flow was measured by R&Disound Doppler as previously validated (26). A muscle biopsy was then obtained from vastus lateralis by use of the percutaneous biopsy technique with suction and immediately frozen in liquid nitrogen. The subjects then performed two-legged dynamic knee-extensor exercise at ~55% of their maximal workload for 180 min (mean workload = 93 ± 4 W). Blood samples and concomitant femoral arterial blood flow measures were obtained at 60, 120, and 180 min during exercise. In addition, further muscle samples were obtained at 30, 90, and 180 min during exercise. Subjects were permitted to drink water ad libitum during exercise but were not permitted to consume any food or drink.

Tissue Analysis

Femoral arterial venous plasma was analyzed for IL-6 and TNF-α by a commercially available enzyme-linked immunosorbent assay (ELISA; R&D Systems Europe, Oxon, UK). All measurements were performed in duplicate, and high-sensitivity kits were used. According to information provided by R&D Systems, the kits used for measuring IL-6 and TNF-α are insensitive to the addition of the recombinant forms of the soluble receptors (sIL-6R, sTNF-R1, and sTNF-R2, respectively); these measurements, therefore, correspond to both soluble and receptor-bound cytokines.

Upon collection, muscle samples were divided into two portions. One portion was analyzed for glycogen, as previously described (33). The second portion was analyzed for IL-6 and TNF-α mRNA. The muscle was extracted for total RNA with a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction of Chomczynski and Sacchi (7), as previously described (24). Final RNA pellets were dissolved in 0.1 mM EDTA (2 μl/mg original wet wt). Reverse transcription reactions were carried out on 22 μl of sample with the superscript II RNAse H⁻ Reverse Transcriptase (Invitrogen) in a reaction volume of 40 μl. All samples were diluted in 160 μl of nuclear-free water.

Real-time PCR was employed to quantify human IL-6 and TNF-α gene expression from the cDNA samples. Both human IL-6 and TNF-α were designed ( Primer Express version 1.0 Applied Biosystems) from the human IL-6 and TNF-α gene sequences (GenBank/EMBL accession nos. M54894 and M36669 and X02910 and X02159 for IL-6 and TNF-α, respectively). An 81-base-length IL-6 fragment was amplified using the forward primer 5’-GGTACATCTCGTCG-GGCATC-3’ and reverse primer 5’-GTGCCCTTTGTGCGTTTCA-3’ (Sigma Geno-sys, Castle Hill, NSW, Australia). A TaqMan fluorescent probe, 5’-FAM (6-carboxyfluorescein)-TGGTACTCTTGTTCATGTCCTTCTCAGGGCTC-3’ TAMRA (6-carboxy-tetramethylrhodamine) (Applied Biosystems) was included with the primers in each reaction. For TNF-α, an 81-base-length fragment was amplified using the
forward primer 5'-CCCAGGCAGTCAGATCATCTTC-3' and reverse primer 5'-AGCTGCCCTACAGCTTTGA-3' (Sigma Geno-sys). A TaqMan fluorescent probe 5'-FAM (6-carboxy-fluorescein)-TCTTCAAGGCCAAGGGCTGCCC-3' TAMRA (6-carboxy-tetramethylrhodamine) (Applied Biosystems) was included with the primers in each reaction. We also amplified 18S mRNA, and the TaqMan probes and primers for this gene were supplied in a control reagent kit (Applied Biosystems). We quantitated gene expression with a multiplex fluorescent probe 5'-HEX (6-carboxytetramethylrhodamine)-TCTTCAAGGGCCAAGGCTGCCC-3' (Molecular Probes) and a single tube, where the primers for 18S were limited to ensure that adequate amounts of reagents were present for amplification of both genes.

It was possible to detect 18S in the same tube as our gene of interest because the reporter dyes attached to the TaqMan probes fluoresce at different emission wavelength maxima. In preliminary experiments, we determined the relative efficiency of amplification of 18S vs. IL-6 or TNF-α. These experiments revealed approximately equal efficiencies of 18S and IL-6 or TNF-α amplifications over different starting template concentrations. We also performed experiments to demonstrate that nonspecific multiplex experiments had no effect on C_T values, as well as primer-limited multiplex 18S vs. nonprimer-limited nonmultiplex 18S reactions. Finally, we determined the linear dynamic range for starting template concentrations.

PCR reactions were carried out in 25-μl reactions of Taq-Man universal PCR master mix (1×), 50 nM TaqMan 18S probe, 20 nM 18S forward primer, 80 nM 18S reverse primer, either 100 nM TaqMan IL-6 probe or 175 nM TaqMan TNF-α probe, 900 nM IL-6 forward primer or 900 nM TNF-α forward primer, 300 nM IL-6 reverse primer, or 900 nM TNF-α reverse primer. Each reaction was made up to volume with RNase-free H2O. Concentrations of the IL-6 and TNF-α probes and primers were chosen on the basis of pilot analyses in which optimal concentrations were determined. Fifty nanograms of cDNA and control preparations were amplified using the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For each sample, a change in (ΔC_T) value was obtained by subtracting 18S C_T values from IL-6 or TNF-α, with the resting value as the control. Resting values for each subject were subtracted from the exercise samples for each subject to derive a ∆ΔC_T value. The expressions of human IL-6 and TNF-α were then evaluated by 2^−∆ΔC_T.

**Statistical Analysis**

All parameters were tested for normalcy in distribution. Accordingly, both IL-6 and TNF-α mRNA values, as well as plasma IL-6, were not normally distributed. Hence, the data for these parameters were log transformed before analyses. A one-way analysis of variance (ANOVA) was performed on the data. After a significant F-test, pair-wise differences were identified using Newman-Keuls post hoc procedure. The significance level was set at P < 0.05. Data are presented as means ± SE unless otherwise stated.

**RESULTS**

Muscle glycogen averaged 398 ± 52 mmol/kg dry wt and progressively decreased (P < 0.05) throughout exercise such that values were 153 ± 50 mmol/kg dry wt after 180 min (Fig. 1). Plasma glucose did not change during the exercise, whereas plasma free fatty acids (FFA) increased (P < 0.05) (Table 1).

We were able to detect both TNF-α and IL-6 mRNA in the resting muscle biopsy samples. Although there was a tendency (P = 0.08) for an increase in TNF-α mRNA when exercise values were compared with those collected before exercise, results were not significant (Fig. 2). In contrast, IL-6 mRNA increased (P < 0.05) after only 30 min of exercise and peaked at the cessation of exercise, when values were ~100-fold higher compared with rest (Fig. 2).

Arterial plasma TNF-α concentration averaged 2.4 ± 0.2 pg/ml at rest and did not increase during exercise. Furthermore, we could not detect any arterial-femoral venous difference for TNF-α; therefore, there was no net release of TNF-α either before or during exercise (Fig. 3). In contrast, arterial concentrations of IL-6 averaged 0.57 ± 0.18 pg/ml at rest but increased (P < 0.05) progressively throughout exercise. In addition, we observed an arterial-femoral venous difference (P < 0.05) for IL-6 after 120 min of exercise, and this remained until the cessation of exercise. As a consequence, we observed net leg release (P < 0.05) of IL-6 after 120 min of exercise, and this persisted until the cessation of exercise (Fig. 3).

Leg blood flow averaged 0.35 ± 0.07 l/min at rest and increased (P < 0.05) to an average of 2.18 ± 0.09 l/min during exercise (Fig. 4).

**Table 1. Plasma glucose and FFA before, during, and after exercise**

<table>
<thead>
<tr>
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<th>Pre</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
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<tbody>
<tr>
<td>Glucose, mmol/l</td>
<td>5.6 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>5.2 ± 0.3</td>
<td>4.8 ± 0.4</td>
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<tr>
<td>FFA, mmol/l</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.3 ± 0.1*</td>
<td>1.9 ± 0.2*</td>
</tr>
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Values are concentrations of glucose and free fatty acids (FFA) (means ± SE; n = 6) before (Pre), during (60 and 120 min), and after (180 min) 3 h of 2-legged knee-extensor exercise. *Significant (P < 0.05) difference from Pre.

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**Fig. 1. Muscle glycogen concentration before (Pre), during (30 and 90 min), and after (180 min) 3 h of two-legged knee-extensor exercise. Values are means ± SE (n = 6 subjects).**
DISCUSSION

The results from this study demonstrate that, despite 3 h of strenuous exercise, contracting muscle increases gene expression and protein release of IL-6, but not of TNF-α. Therefore, our data demonstrate a clear disassociation between IL-6 and TNF-α production in skeletal muscle during exercise and are consistent with the hypothesis that TNF-α, but not IL-6, impairs glucose uptake by skeletal muscle.

Although TNF-α has been previously measured in human skeletal muscle (22), this is the first study to measure the effect of acute contraction on TNF-α in human muscle. These data support those of Greiwe et al. (10), who found that TNF-α gene and protein expression decreased in frail elderly patients after a resistance training program. In the present study, despite 3 h of continual contractile activity that resulted in a marked decrease in intramuscular glycogen (Fig. 1), TNF-α mRNA was not significantly increased compared with rest. It could be argued that TNF-α mRNA and protein release did not increase because the glycogen levels were not reduced to very low levels and that TNF-α may be sensitive only to very low glycogen stores. Of note, however, there was an approximately fourfold increase (P = 0.08) in TNF-α mRNA after 30 min of exercise, after which time it did not increase any further. If TNF-α were sensitive to glycogen content, one would not have expected the greatest degree of change, albeit not statistically significant, to be observed after 30 min of exercise when glycogen stores were hardly compromised. In contrast, the degree of increase in IL-6 mRNA peaked after 180 min of exercise. We have been able to demonstrate, in preliminary experiments, that TNF-α gene expression is inducible in human skeletal muscle cells. We have shown that, when stimulated with the calcium ionophore ionomycin, primary human muscle cells in culture significantly increased TNF-α gene expression after 6 h of incubation, after which time it fell to basal levels (Keller C, Hellsten Y, Pilegaard H, Febbraio M, and Pedersen BK, unpublished data). Therefore, we cannot rule out the possibility that more intense exercise, which would increase the magnitude of calcium release from the sarcoplasmic reticulum, would in turn increase TNF-α gene expression. However, it would appear from our cell culture experiments that any increase would be transient. In contrast to the pattern of TNF-α expression, IL-6 peaked in the same stimulated culture preparation after 24 h (13). Given these preliminary results and those from the present experiment, we propose that one function of the marked increase in IL-6 gene expression in skeletal muscle during muscle contraction may be to inhibit any in-
increase in TNF-α production. During contraction, muscle glucose uptake is markedly increased compared with rest; therefore, our hypothesis is consistent with the observation that TNF-α impairs glucose disposal in skeletal muscle (42). Indeed, there are data demonstrating that IL-6 can impair increases in TNF-α. Tanaka et al. (35) recently demonstrated that, during viral myocarditis, the serum TNF-α concentration is markedly reduced in transgenic mice, which overexpress IL-6 compared with wild-type mice. 

Along with the differential expression of IL-6 and TNF-α mRNA within skeletal muscle, we also observed that IL-6, but not TNF-α, was released during contraction. In the current study, we did not make protein measures within the skeletal muscle, and therefore we cannot be certain that TNF-α protein was not expressed in contracting skeletal muscle. However, given the fact that TNF-α mRNA did not significantly increase within the muscle, this possibility appears remote. It is also important to note that arterial TNF-α concentration did not increase as a result of exercise, and therefore we cannot rule out the possibility that, in the studies in which TNF-α increased in the blood, the muscle was indeed the source of this increase. On careful examination of the literature, however, elevations in plasma TNF-α as a result of exercise have been observed only after marathon running (20, 21, 32, 37, 38) and not after other forms of strenuous or prolonged exercise. During marathon running, a decrease in blood flow to the splanchnic bed may occur that can induce an ischemic state, resulting in gut wall bacterial translocation (3). Indeed, endotoxemia has been observed after marathon running (6), and because endotoxins are lipopolysaccharides of gram-negative bacteria, it is probable that the increased plasma TNF-α observed during marathon running is likely to result from systemic endotoxemia rather than from contracting muscle release. In addition, we have recently observed that, during eccentric exercise that results in severe muscle membrane damage, TNF-α levels are not increased (38). These data lend support to the suggestion that TNF-α is enhanced in the plasma only after marathon running and that this is due to endotoxemia.

Our observation of IL-6 gene expression within, and protein release from, contracting skeletal muscle is consistent with our previous observations (14, 31, 33, 34). Although we have not yet determined the precise biological action of this phenomenon, we have hypothesized that the role of IL-6 release from contracting muscle during exercise is to act in a hormone-like manner to mobilize extramuscular substrates and/or augment substrate delivery during exercise (23). Of note, Wallenius et al. (40) recently demonstrated that IL-6-deficient mice develop obesity and glucose intolerance, whereas chronic treatment of these animals with IL-6 partially attenuates these metabolic perturbations. Because the kinetics of IL-6 gene expression differed markedly from those of TNF-α, these present data might also suggest that another role for muscle-derived IL-6 is to impair TNF-α expression in skeletal muscle.

Indeed, previous studies provide evidence for the notion that a primary role of IL-6 may be to inhibit the production of TNF-α. IL-6 inhibits LPS-induced TNF-α production in both cultured human monocytes and the human monocytic line U937 (30). The suppressive effect occurs at the level of transcription in human peripheral blood mononuclear cells. In in vivo endotoxin models, levels of TNF-α were elevated in anti-IL-6-treated mice (17) and in IL-6-deficient knockout mice compared with control mice, suggesting that circulating IL-6 regulates TNF-α levels (18). IL-6 has strong anti-inflammatory effects. Thus IL-6 administration in humans induces interleukin-1 receptor antagonist (IL-1ra) and soluble TNF-α receptors, but not IL-1β and TNF-α (36). Furthermore, IL-6 induces the production of C-reactive protein, which has a role both in the induction of anti-inflammatory cytokines in circulating monocytes and in the suppression of the synthesis of proinflammatory cytokines in tissue macrophages (25). Taking these observations together, we propose that muscle contractions result in the generation of a strong anti-inflammatory response.

Both exercise and insulin stimulation increase glucose uptake in skeletal muscles (27, 28). Two key proteins involved in, respectively, insulin-stimulated and contraction-induced increased glucose uptake are phosphatidylinositol 3 (PI 3)-kinase and mitogen-activated protein kinase (MAPK) (28). IL-6 can bind to either a soluble IL-6 receptor or a membrane-bound receptor and activate the gp130-signaling pathway (41). Stimulation of the gp130 receptor in cardiac muscle increases the activity of both PI 3-kinase and MAPK (41). Importantly, insulin resistance is associated with a decreased activity of PI 3-kinase and MAPK (41). Stimulation of the gp130 receptor in cardiac muscle increases the activity of both PI 3-kinase and MAPK (41). Importantly, insulin resistance is associated with a decreased activity of PI 3-kinase and MAPK (41). Stimulation of the gp130 receptor in cardiac muscle increases the activity of both PI 3-kinase and MAPK (41). Importantly, insulin resistance is associated with a decreased activity of PI 3-kinase and MAPK (41).
downregulate both PI 3-kinase and MAPK in skeletal muscle cells (9). However, in principle, PI 3-kinase and MAPK may also regulate IL-6; therefore, a possible relationship could be inverse. Thus MAPK, in particular p38, can activate IL-6 (8), whereas PI 3-kinase has an inhibitory effect on IL-6 expression, since pharmacological blockade of PI 3-kinase can enhance monocyte IL-6 production severalfold (4). This implies that increased MAPK activity during exercise would stimulate the IL-6 production. Furthermore, because of the reduced activity of PI 3-kinase in insulin-resistant muscles, IL-6 expression would be increased in these muscles.

In conclusion, we have demonstrated for the first time a clear disassociation between IL-6 and TNF-α production in skeletal muscle during exercise. We have shown that, although intramuscular IL-6 gene expression and protein release are remarkable during continuous contractile activity, no such response is evident for TNF-α. Hence, our data are consistent with the hypothesis that TNF-α, but not IL-6, impairs glucose uptake by skeletal muscle.

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