Upregulation of IL-6 mRNA by IL-6 in skeletal muscle cells: role of IL-6 mRNA stabilization and Ca²⁺-dependent mechanisms

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First published July 5, 2007; doi:10.1152/ajpcell.00142.2007.—Skeletal muscle cells have been established as significant producers of IL-6 during exercise. This IL-6 production is discussed as one possible mediator of the beneficial effects of physical activity on glucose and fatty acid metabolism. IL-6 itself could be the exercise-related factor that upregulates and maintains its own production. We investigated this hypothesis and the underlying molecular mechanism in cultured C₂C₁₂ cells. IL-6 led to a rapid and prolonged increase in IL-6 mRNA, which was also found in human myotubes. Because IL-6 has been shown to act as an autocrine factor upregulating its mRNA expression, we hypothesized that signal transduction via IL-6 activates AMP-activated kinase (AMPK) during exercise. Pharmacological activation of AMPK with 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside upregulated IL-6 mRNA expression, which was blocked by knockdown of AMPK α₁ and α₂ using small, interfering RNA (siRNA) oligonucleotides. However, the effect of IL-6 was shown to be independent of AMPK, since the siRNA approach silencing the AMPK α-subunits did not reduce the upregulation of IL-6 induced by IL-6 stimulation. The self-stimulatory effect of IL-6 partly involves a Ca²⁺-dependent pathway. IL-6 increased intracellular Ca²⁺, and intracellular blockade of Ca²⁺ with a Ca²⁺ chelator reduced the IL-6-mediated increase in IL-6 mRNA levels. Moreover, inhibition of Ca²⁺/calmodulin-dependent kinase kinase with STO-609 or the siRNA approach decreased IL-6 mRNA levels of control and IL-6-stimulated cells. A major, STO-609-independent mechanism is the IL-6-mediated stabilization of its mRNA. The data suggest that IL-6 could act as autocrine factor upregulating its mRNA levels, thereby supporting its function as an exercise-activated factor in skeletal muscle cells.

5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside; AMP-activated kinase; STO-609; calcium/calmodulin-dependent kinase kinase; C₂C₁₂ cells

DURING THE PAST DECADE, THE skeletal muscle has been established as a significant producer of IL-6 in noninflammatory conditions. Several studies in exercising humans have demonstrated that muscle contraction per se is a major stimulus for de novo synthesis of IL-6 mRNA and protein and that the myocytes are the source of the produced IL-6 (18). The induction of IL-6 mRNA expression is very rapid, and a 10- to 20-fold increase had been found after only 30 min of exercise with an increasing progression when the exercise performance proceeds (25). The IL-6 mRNA levels peaked immediately after exercise but remained elevated during the next 20 h compared with values at rest (34). This upregulation after the cessation of the stimulus was even more prominent at the protein level as demonstrated by IL-6 immunostaining of human muscle fibers (34). Notably, in contrast to chronically elevated circulating IL-6 plasma levels, which are implicated in the development of insulin resistance (11, 36), this exercise-induced IL-6 expression is discussed as a benign modulator of muscle metabolism (10). IL-6 enhances glucose uptake and fatty acid oxidation in skeletal muscle cells and promotes insulin action (6, 7, 35, 39). Thus the IL-6 production induced by physical activity could be involved in the beneficial effects of exercise on insulin sensitivity.

The regulation of IL-6 gene activation and expression in inflammatory conditions has been studied in detail (29, 31), including in studies of skeletal muscle cells (12, 13). In contrast, the molecular regulation of IL-6 expression during exercise is less clear. Muscle contraction activates several signaling pathways and exercise-related factors, which are therefore possible mediators that lead to enhanced IL-6 expression. Among them, IL-6 itself is an interesting candidate. At least in skeletal muscle under low-glycogen conditions, the IL-6 release occurs before the increase in IL-6 gene transcription, indicating that this early release must be due to IL-6 protein storage in the muscle (30). The secreted IL-6 could then, in a positive feedback loop, activate its own expression. An autocrine regulation of IL-6 production was postulated because, after infusion of recombinant IL-6 in humans, substantial IL-6 mRNA expression was observed in skeletal muscle (26).

The rapid and transient activation of the AMP-activated kinase (AMPK) during exercise could also be important for IL-6 production (16, 30, 32). Individual values of AMPK activity and IL-6 release correlated significantly over a 60-min trial on a bicycle ergometer (30). Pharmacological activation of AMPK with the AMP analog 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) activated IL-6 expression in fibroblasts (9). In addition, activation of AMPK by IL-6 was demonstrated in skeletal muscle in vivo and in cell cultures (2, 7, 27). We hypothesized that, in turn, signal transduction via AMPK during exercise could lead to enhanced IL-6 expression.

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Intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) could also play an important role in exercise-related IL-6 expression. Muscle contraction induces Ca\(^{2+}\) release from the sarcoplasmic reticulum (5), and Ca\(^{2+}\) is an important regulator of IL-6 expression, as demonstrated by stimulation of rat soleus muscle, human skeletal muscle cells, or L6 myotubes with the Ca\(^{2+}\) ionophore ionomycin (8, 20, 24) or with depolarization-induced slow Ca\(^{2+}\) transients in C\(_2\)C\(_1\)2 cells (23). However, a causal relationship linking exercise-related Ca\(^{2+}\) signaling to the enhanced IL-6 expression has not been shown (1, 19).

In the present study, we examined the regulation of IL-6 expression in skeletal muscle cells by IL-6, AMPK, and Ca\(^{2+}\). Using C\(_2\)C\(_1\)2 cells, we demonstrated a self-stimulatory effect of IL-6, which is AMPK independent and is mediated via a Ca\(^{2+}\)-dependent signaling pathway and stabilization of IL-6 mRNA.

**EXPERIMENTAL PROCEDURES**

**Materials.** C\(_2\)C\(_1\)2 cells were from American Type Culture Collection (Manassas, VA). Cell culture media and supplements were from Cambrex (Verviers, Belgium); phosphatase inhibitors, human recombinant IL-6, SB-203580, actinomycin D, STO-609, cyclosporin A, ionomycin, and BAPTA-AM were from Sigma (Munich, Germany); mouse recombinant IL-6 was from R\&D Systems (Wiesbaden, Germany); light cycler system was from Roche (Mannheim, Germany); fura 2-AM was from Invitrogen (Karlsruhe, Germany); and AICAR was from Chemos (Regenstauf, Germany). Antibodies against phospho-STAT-3 (Tyr705), STAT-3, AMPK \(\alpha\), phospho-acetyl-CoA carboxylase (ACC) (Ser79), phospho-p38 MAPK, and p38 MAPK were from Cell Signaling (Frankfurt, Germany); antibodies against AMPK \(\alpha\), AMPK \(\alpha\) \(_2\), and ACC were from Upstate Biotechnology (Lake Placid, NY); antibodies against Ca\(^{2+}\)/calmodulin-dependent kinase kinase \(\alpha\) (pan) (CaMKK) were from BD Biosciences (Heidelberg, Germany); and antibodies against CaMKK \(\alpha\) and \(\beta\) were from Santa Cruz (Santa Cruz, CA).

**Cell culture.** C\(_2\)C\(_1\)2 myoblasts were cultured in DMEM containing 25 mM glucose, 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Stimulation was performed in DMEM containing 5.5 mM glucose and 2% FCS. Human primary myotubes were cultured and stimulated as recently described (28, 38).

**RT-PCR and real-time quantitative PCR analysis.** Reverse transcription of total RNA (1 \(\mu\)g) was performed in a volume of 20 \(\mu\)l using random hexamers and avian myeloblastosis virus reverse transcriptase with the first-strand cDNA synthesis kit for RT-PCR (Roche) as described (38). Aliquots (2 \(\mu\)l) of the reverse transcription reactions were then submitted in duplicate to online quantitative PCR with the Light Cycler system (Roche) with SYBR green using FastStart DNA-Master SYBR green I (Roche). For human IL-6, the following primer pairs were used: sense, ccagctatgaactccttctc; antisense, gctgttgtcacatctctc, product of 425 bp. For mouse IL-6, the following primer pairs were used: sense, ccagctatgaactccttctc; antisense, gctgttgtcacatctctc, product of 425 bp. For human IL-6, cycling was performed by denaturing at 95°C for 15 s, annealing at 63°C for 10 s, and elongation for 17 s (the number of cycles was 45). For mouse IL-6, annealing was at 65°C for 5 s and elongation was for 11 s (the number of cycles was 50). For mouse SOCS-3, annealing was at 66°C for 10 s, elongation was for 9 s, and the number of cycles was 45. For mouse \(\beta\)-actin, annealing was at 69°C for 10 s, elongation was for 10 s, and the number of cycles was 45.

**Small interfering RNA.** Small interfering RNA (siRNA) oligonucleotides targeting mouse AMPK \(\alpha\), mouse CaMKK \(\alpha\), and CaMKK \(\beta\) were designed, synthesized, and annealed at Dharmacon Research (Lafayette, CO). For siRNA targeting the mouse AMPK \(\alpha\), mRNA, the sequence AGAAGUGUGAGAAUGUCG (nucleotides 882–900, GenBank accession no. Y1885266) was used. An unrelated siRNA targeting firefly luciferase was used as control in all experiments. Transfection was performed with CellPect (Amersham Biosciences, Buckinghamshire, UK) with 200 nM siRNA according to the instructions of the manufacturer. Briefly, 1 \times 10\(^5\) cells/well were seeded in six-well plates and transfected in DMEM containing 25 mM glucose and 10% FCS without antibiotics. Twenty-four hours after the glycero1 shock, cells were stimulated as indicated.

**Measurement of [Ca\(^{2+}\)]\(_i\).** [Ca\(^{2+}\)]\(_i\) was measured at 37°C in single cells by the fura 2 method according to Grynkiewicz et al. (15) using equipment and software from TILL photonic (Gräfelfing, Germany). C\(_2\)C\(_1\)2 cells were loaded with a mixture of fura 2-AM (5 \(\mu\)M) and Fluo-4 (0.2 \(\mu\)M) for 45 min at 37°C. Intracellular fura 2 was excited alternately at 340 or 380 nm by means of an oscillating diffraction grating. The excitation light was directed through the objective (PlanNeofluor \(\times 40\) objective; Zeiss, Stuttgart, Germany) by a glass fiber light guide and a dichroic mirror. The emitted light was filtered (LP 515 nm) and measured by a digital camera. The ratio of the emitted light intensity at 340-to-380 nm excitation was used to monitor changes in [Ca\(^{2+}\)]\(_i\). Values are given as arbitrary units (changes in 340-to-380 nm fluorescence ratio).

**Western blotting.** Cell lysis and Western blotting were performed as previously described (40).

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**Fig. 1.** IL-6 induces IL-6 expression. C\(_2\)C\(_1\)2 myocytes were stimulated with IL-6, which is AMPK independent and is mediated via a Ca\(^{2+}\)-dependent signaling pathway and stabilization of IL-6 mRNA.

**A**

**B**

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**Table 1.** Real-time PCR analysis of IL-6 expression in C\(_2\)C\(_1\)2 cells. Relative expression of IL-6 mRNA was determined by real-time PCR. Relative expression was calculated using the 2\(^{-}\)\(\Delta\Delta C_{T}\) method. Results are means \(\pm\) SE of at least 4 separate experiments. mRNA expression of untreated cells was set as 1.

<table>
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<tr>
<th>Condition</th>
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**Legend:** *P < 0.05 vs. control.
Statistical analysis. Results presented are derived from at least three independent experiments. Means ± SE were calculated, and groups of data were compared with Student’s *t*-test. Statistical significance was set at *P* < 0.05.

RESULTS

IL-6 increases its mRNA levels. First, we studied whether incubation of C2C12 cells with IL-6 could increase IL-6 mRNA levels. We found a stimulatory effect using 20 ng/ml IL-6 with no further increase after incubation with 100 ng/ml (Fig. 1A). This upregulation is rapid and found first after 2 h of IL-6 incubation, with a further increase after 16 and 24 h (Fig. 1B). To achieve maximum stimulation in subsequent experiments, we used 20 ng/ml IL-6 for 24 h. The stimulatory effect of IL-6 on its own expression was also observed in human myotubes, indicating that the effect of IL-6 is independent of the differentiation status of the skeletal muscle cells (data not shown).

Activation of AMPK by AICAR upregulates IL-6 expression. Because AMPK has been verified as a downstream mediator of IL-6 effects in skeletal muscle cells, we clarified whether activation of AMPK could in turn increase IL-6 mRNA expression in C2C12 cells. Incubation with 0.5 mM of the pharmacological activator AICAR caused a more than three-fold stimulation of IL-6 mRNA expression, which was further increased with treatment with 1 mM AICAR (Fig. 2A). The first relevant increase in IL-6 expression was found after 4 h of treatment with AICAR, with a more pronounced effect after 24 h (Fig. 2B). In further experiments, C2C12 cells were stimulated with 1 mM AICAR for 24 h. In human myotubes,
stimulation with 1 mM AICAR for 24 h resulted in a 2.6-fold upregulation of IL-6 mRNA levels (data not shown).

To verify the causal relation of AMPK activation on IL-6 mRNA expression, we silenced AMPK α-subunit expression using the siRNA approach. Transfection of siRNA oligonucleotides targeting α1-subunit resulted in a strong reduction of the corresponding protein (Fig. 2C). The expression of the less prominent α2-subunit was decreased when siRNA oligonucleotides against this subunit were used (Fig. 2C). Knockdown of AMPK α1-subunit was sufficient to almost completely prevent the AICAR-induced upregulation of IL-6 mRNA expression with no reduction of basal IL-6 mRNA levels (Fig. 2D). The additional knockdown of the α2-subunit had no further effect on IL-6 expression. Thus the effect of AICAR is mediated via AMPK, and AMPK activation leads to enhanced IL-6 expression.

The self-induced upregulation of IL-6 expression is independent of AMPK. Because the pharmacological activation of AMPK was sufficient to induce IL-6 expression (Fig. 2), we studied the participation of AMPK in autocrine IL-6 upregulation. Knockdown of AMPK α1-subunit completely prevented basal, IL-6-induced, and AICAR-induced phosphorylation of the AMPK substrate ACC on Ser79 (Fig. 3A), demonstrating almost complete inhibition of AMPK activity by knockdown of AMPK α1-subunit. The IL-6-induced phosphorylation of STAT-3 was not reduced by silencing AMPK, indicating that the IL-6-dependent Janus kinase/STAT pathway was not inhibited (Fig. 3A). Moreover, the increase in IL-6 expression after incubation with IL-6 was not affected by knockdown of AMPK α1- or α2-subunit (Fig. 3B). These data indicate that the self-induced stimulation of IL-6 expression is not mediated via AMPK activation.

Regulation of IL-6 expression by the p38 MAPK pathway. The p38 MAPK pathway has been implicated in the regulation of IL-6 expression during exercise (8). Therefore, we studied the involvement of this pathway in the upregulation of IL-6 expression by IL-6. Because the p38 MAPK pathway has been shown to mediate downstream effects of AMPK activation (9, 44), we also studied this pathway in AICAR-treated C2C12 cells.

Stimulation with AICAR for 60 and 240 min resulted in enhanced phosphorylation of p38 MAPK, demonstrating the activation of this pathway by the AMPK activator (Fig. 4A). The stimulation with IL-6 led to a more rapid and transient phosphorylation of p38 MAPK (Fig. 4B). Inhibition of the p38 MAPK pathway with SB-203580 clearly reduced IL-6 mRNA expression in control, AICAR-treated, and IL-6-treated cells (Fig. 4, C and D). In contrast to the remaining relative increase in IL-6 levels found in IL-6-treated cells (Fig. 4D), the effect of AICAR was completely blocked in the presence of...
10 μM of the inhibitor (Fig. 4C). Thus the AICAR-induced activation of AMPK leads to enhanced IL-6 expression via the p38 MAPK pathway, whereas the effects of IL-6 are at least partially independent of the activity of this pathway.

Role of Ca²⁺-dependent signaling in the self-stimulatory effect of IL-6. Next, we investigated the possible involvement of Ca²⁺-dependent signaling, which has been demonstrated to be activated by exercise and to be important for IL-6 expression (5, 7, 20).

First, we studied the effect of IL-6 on [Ca²⁺], (Fig. 5, A and B). The majority of the cells displayed oscillations of [Ca²⁺], under control conditions, as shown in the initial phase (Fig. 5A). Addition of IL-6 (20 ng/ml) was followed by a reversible increase of [Ca²⁺]. In 32% of all cells tested, IL-6 application induced a rapid Ca²⁺ peak and a second phase in which [Ca²⁺], remained elevated above basal values (Fig. 5A). In the other cells, there was no peak increase but the shape of oscillations changed, and overall [Ca²⁺], was larger than under control conditions. Addition of ATP, which has been reported as inducer of [Ca²⁺], in skeletal muscle cells (46), resulted in a similar pattern with a more pronounced, rapid Ca²⁺ peak (Fig. 5A). To quantify the effect of IL-6 on [Ca²⁺], the area under the curve (AUCF340/380, where F340/380 is the 340-to-380 nm fluorescence ratio) was calculated for 2 min before and immediately after the bath solution was changed, respectively (Fig. 5B). AUCF340/380 was 2,352 ± 170 arbitrary units × min under control conditions and raised to 8,626 ± 1,201 arbitrary units × min in the presence of IL-6 (n = 50, P < 0.001).

A first hint for a possible involvement of the increase in [Ca²⁺], in the IL-6-induced upregulation of IL-6 expression was the partial reduction by the intracellular chelator of Ca²⁺, BAPTA (1.77 ± 0.32 vs. 2.40 ± 0.47; Fig. 5C). In contrast, when extracellular Ca²⁺ was blocked with 0.5 mM EGTA, the increase in IL-6 mRNA levels was not prevented (data not shown).

Role of calcineurin and CaMKK in IL-6 expression. The upstream CaMKK-α and β-subunits and the serine-threonine phosphatase calcineurin respond to exercise-induced elevation of [Ca²⁺], (14, 37, 43) and have been implicated in Ca²⁺/-calmodulin-dependent gene expression during muscle contraction (3, 4, 22, 41). Inhibition of calcineurin with 1 or 5 μM cyclosporin A had no effect on IL-6 mRNA expression in IL-6-treated cells (Fig. 6A). To evaluate the role of CaMKK, we used the CaMKK inhibitor STO-609. We found a significant reduction of IL-6 expression below values of untreated control cells with 2.5 and 10 μg/ml of STO-609 in both unstimulated and IL-6-stimulated cells (Fig. 6B). The concentrations of STO-609 applied in these experiments, although a widely used concentration to study specific effects of CaMKK, could, besides CaMKK, partly inhibit the activity of other kinases, most notably AMPK (17). The participation of this kinase, however, was clearly excluded by the siRNA approach (Fig. 3). A direct inhibition of the IL-6 signaling cascade by STO-609 could also be ruled out because the IL-6-induced phosphorylation of STAT-3 and the induction of SOCS-3 expression were not influenced by this inhibitor (Fig. 6, C and D). To obtain specific inhibition of CaMKK, siRNA oligonucleotide-mediated knockdown was applied. Silencing of CaMKK-α and β-isoforms with isoform-specific oligonucleotides was verified by Western blotting (Fig. 6E). The knockdown of CaMKK α or CaMKK β-isoform alone had no effect on IL-6-induced IL-6 expression, whereas knockdown of both isoforms clearly reduced the IL-6-stimulated increase by 30% (Fig. 6F). The inhibition of CaMKK β alone and of both isoforms also reduced basal IL-6 expression (Fig. 6F).

Thus the data suggest a role for Ca²⁺-dependent pathways in basal and self-induced stimulation of IL-6 expression. To test
Fig. 6. Role of calcineurin and Ca\textsuperscript{2+}/calmodulin-dependent kinase (CaMKK) in IL-6 expression. Cells were preincubated with the indicated concentrations of cyclosporin A (CsA; A) or STO-609 (B) for 60 min before 20 ng/ml IL-6 was added for a further 24 h. IL-6 mRNA expression of untreated cells was set as 1. * $P < 0.05$ vs. unstimulated cells; # $P < 0.05$ vs. cells without inhibitor stimulated with IL-6. C: cells were preincubated with 10 µg/ml STO-609 for 60 min before 20 ng/ml IL-6 was added. Cytosolic extracts were separated by SDS-PAGE and immunoblotted with anti-phospho-STAT-3 and anti-STAT-3 antibodies. D: cells were preincubated with 10 µg/ml STO-609 for 60 min before 20 ng/ml IL-6 was added for a further 24 h. SOCS-3 mRNA expression of untreated cells was set as 1. * $P < 0.05$ vs. unstimulated cells. E: C2C12 cells were transfected with siRNA oligonucleotides targeting CaMKK- and -isoforms or firefly luciferase as control (−). Forty hours after transfection, cells were harvested and cytosolic extracts were separated by SDS-PAGE and immunoblotted with anti-CaMKK (pan-specific) antibodies. Immunodetection with isoform-specific antibodies gave similar results (data not shown). F: 40 h after transfection, cells were stimulated with 20 ng/ml IL-6 for 24 h, and IL-6 mRNA expression was measured by real-time PCR. Results are means ± SE of 4 separate experiments. mRNA expression of si-con-transfected, untreated cells was set as 1. * $P < 0.05$ vs. unstimulated cells; # $P < 0.05$ vs. stimulated cells. G: C2C12 myocytes were stimulated with ionomycin (iono), IL-6, or both for 8 h, and IL-6 mRNA expression was measured by real-time PCR. Results are means ± SE of at least 4 separate experiments. mRNA expression of untreated cells was set as 1. * $P < 0.05$ vs. control; § $P < 0.05$ vs. IL-6 alone.
the effect of IL-6 in the presence of a strong activator of [Ca^{2+}], we cotreated the cells with IL-6 and the calcium ionophore ionomycin. Because incubation with ionomycin for 24 h clearly reduced cell proliferation and viability (data not shown), we used 8 h of stimulation. Under these conditions, 1 μM ionomycin induced IL-6 expression 4.1-fold compared with the 2.6-fold induction by IL-6 (Fig. 6G). Although 0.1 μM ionomycin alone had no stimulatory effect, the combination with IL-6 resulted in a greater induction than IL-6 alone (3.4 ± 0.30 compared with 2.6 ± 0.24; Fig. 6G). The additive effect of 1 μM ionomycin and IL-6 was even more pronounced (10.5 ± 1.5-fold increase; Fig. 6G). Although these data underline the importance of [Ca^{2+}], for an enhanced IL-6 expression, they also suggest the involvement of other, IL-6-induced mechanisms in the IL-6 upregulation.

IL-6 increases mRNA stability. Treatment with the inhibitor of the p38 MAPK pathway (SB-203580), with the CaMKK inhibitor STO-609, and the knockdown of CaMKK clearly reduced the IL-6-mediated expression of IL-6 mRNA; however, a relative increase of IL-6 mRNA levels relative to unstimulated cells cultured under identical conditions remained. Therefore, we hypothesized that IL-6 could lead to increased mRNA levels by stabilizing IL-6 mRNA. An mRNA stability assay was performed in the presence of 5 μg/ml actinomycin D. Preincubation with IL-6 for 30 min clearly increased the amount of the remaining IL-6 mRNA at the different time points studied (Fig. 7A). Calculation of the mRNA half-life of IL-6 under basal conditions revealed a half-life of 35 ± 5 min, which was doubled in the presence of IL-6 (75 ± 17 min). This mRNA-stabilizing effect of IL-6 was not prevented when the assay was performed with 10 μg/ml STO-609; the data in Fig. 7B are presented as IL-6 expression units, which show the STO-609-mediated decrease in absolute mRNA expression levels of IL-6 as expected, whereas the presence of IL-6 delayed the degradation of the mRNA (Fig. 7B). These data indicate that IL-6 has a rapid (within 30 min) stabilizing effect on its mRNA, which is independent of CaMKK.

DISCUSSION

IL-6 has been established as an exercise-related factor expressed and released from the contracting muscle, but the underlying molecular mechanisms for the enhanced IL-6 expression have not been clarified. In the present study, we focused on the role of IL-6 itself, AMPK, and [Ca^{2+}], as regulators of IL-6 expression in skeletal muscle cells.

Our data provided clear evidence that IL-6 stimulates its own expression in skeletal muscle cells. This increase has been demonstrated in C2C12 myoblasts and in human myotubes. It is an early event, first demonstrated after 2 h of IL-6 stimulation with increasing mRNA levels after 16 and 24 h. In vivo data on IL-6 protein levels in human skeletal muscle during and after exercise reported a first rapid increase in IL-6 within minutes after start of the exercise performance, which was discussed as release from intracellular storage pools of IL-6 protein (30). Our data on IL-6 mRNA expression in skeletal muscle cells suggest that this released IL-6 could then act as an autocrine stimulatory factor to enhance its own expression and production, leading to the increased IL-6 mRNA and protein levels found several hours after the exercise bout. At least from our in vitro cell culture data, it could be suggested that IL-6 is an important factor for the enhanced production of IL-6 during and after exercise.

AMPK-dependent signaling appeared to be a promising candidate for mediating the autocrine effects of IL-6. The activation of AMPK by IL-6 has been demonstrated in skeletal muscle cells, including human myotubes and L6 myotubes (2, 7) and using mouse skeletal muscle strips (27). Treatment of mice with IL-6 also resulted in a very rapid phosphorylation of AMPK and its substrate ACC in skeletal muscle (Weigert, unpublished observations). Moreover, the activation of AMPK during exercise correlated with the IL-6 release from the contracting muscle (30), and activation of AMPK using AICAR led to enhanced IL-6 expression in fibroblasts (9). Of note, CaMKK is an upstream AMPK kinase and a CaMKK-dependent AMPK activation was demonstrated in skeletal muscle after increases in [Ca^{2+}] (17, 21, 42). Unexpectedly, however, the self-stimulatory upregulation of IL-6 expression in C2C12 cells was found to be independent of AMPK. The knockdown of AMPK α1- and α2- isoforms did not reduce IL-6 expression, although this approach was suitable to prevent IL-6- and AICAR-induced ACC phosphorylation and the AICAR-induced IL-6 expression. We admit that we could not exclude, based on the cell culture data, that exercise-related
AMPK activation is implicated in the enhanced IL-6 expression in the contracting muscle. However, the data in the present study did not support a role of AMPK in the autocrine effect of IL-6.

It is not clear why the activation of AMPK using AICAR led to increased IL-6 expression, whereas the activation of AMPK by IL-6 is not responsible for the self-stimulatory effect of IL-6. Because the effect of AICAR was prevented by knockdown of AMPK, involvement of other AICAR-activated, AMPK-independent pathways in the enhanced IL-6 expression appears unlikely. It is possible that the intensity and duration of AMPK activity induced by AICAR are more pronounced and thus suitable to increase IL-6 expression, whereas the effect of IL-6 on AMPK activity is not sufficient to support an upregulation.

Moreover, our data provide strong evidence that the self-stimulatory effect of IL-6 is, to a great extent, based on stabilization of IL-6 mRNA. In particular, the first increase in IL-6 mRNA levels found after 2 h in the C2C12 cells could be explained by the rapid effect of IL-6 on its mRNA stability. IL-6 mRNA shares with the mRNA of several interleukins and cytokines the feature of being very unstable; thus delay of mRNA degradation is a common posttranscriptional mechanism to upregulate the expression of these proteins (33). The calculated half-life of IL-6 mRNA in unstimulated C2C12 cells was 35 min, well in line with data reported earlier in other cells (33). This instability is determined by AU-rich elements located in the 3'-untranslated region. The binding of RNA-binding proteins to these sequences regulates the rate of degradation of IL-6 mRNA. In the present study, we did not perform experiments on the mechanism of the IL-6-induced increase in the half-life of its mRNA. Recently, a similar self-stabilizing effect has been reported for IL-4, which is mediated via the RNA-binding protein HuR (45).

A further novel finding is the increase in [Ca\(^{2+}\)], induced by stimulation of the C2C12 cells with IL-6. Because several studies have established that a rise in [Ca\(^{2+}\)], is an important stimulator for IL-6 expression (20, 23, 24), this result could provide a second mechanism for the upregulation of IL-6 mRNA. Additional support for this hypothesis comes from the observation that the calcium chelator BAPTA reduced the self-induced IL-6 expression. Inhibition of CaMKK by STO-609 or by siRNA-mediated knockdown also decreased IL-6 mRNA levels, albeit with different degrees of effectiveness, which might be explained by the remaining CaMKK activity in the siRNA oligonucleotide-transfected cells or by inhibition of other kinases by STO-609. Inhibition of CaMKK additionally reduced basal IL-6 expression. These results indicate that Ca\(^{2+}\), is a strong activator of IL-6 expression. Because Ca\(^{2+}\)-dependent pathways also appeared to be involved in basal regulation of IL-6 expression, the relative effect of the IL-6-induced increase in [Ca\(^{2+}\)], on the self-induced upregulation of IL-6 is difficult to determine. However, the pronounced additive effect of IL-6 and the Ca\(^{2+}\), ionophore ionomycin suggests a major contribution of the IL-6-induced mRNA stability or other mechanisms to the self-stimulatory effect.

In conclusion, the present study provides new information on the possible functions of IL-6 in the working muscle during exercise and emphasizes the role of IL-6 as an exercise factor. In addition to the recently described metabolic properties of IL-6, namely, the improvement of glucose uptake and glycogen synthesis and the activation of fatty acid oxidation (2, 7, 39), our data suggest that IL-6 could enhance and support the increase in [Ca\(^{2+}\)], which is a crucial signal for the adaptive response of the working muscle to exercise (5). Moreover, IL-6 could maintain its activity in a positive feedback loop, leading to enhanced IL-6 expression beyond cessation of exercise performance.

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