Activation of AMP-Activated Protein Kinase by Interleukin-6 in Rat Skeletal Muscle

Association With Changes in cAMP, Energy State, and Endogenous Fuel Mobilization

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OBJECTIVE—Interleukin-6 (IL-6) directly activates AMP-activated protein kinase (AMPK) in vivo and in vitro; however, the mechanism by which it does so is unknown.

RESEARCH DESIGN AND METHODS—We examined this question in skeletal muscle using an incubated rat extensor digitorum longus (EDL) muscle preparation as a tool.

RESULTS—AMPK activation by IL-6 coincided temporally with a nearly threefold increase in the AMP:ATP ratio in the EDL. The effects of IL-6 on both AMPK activity and energy state were inhibited by coinubcation with propranolol, suggesting involvement of β-adrenergic signaling. In keeping with this notion, IL-6 concurrently induced a transient increase in cAMP, and its ability to activate AMPK was blocked by the adenyl cyclase inhibitor 3′,5′-diodeoxyadenosine. In addition, like other β-adrenergic stimuli, IL-6 increased glycogen breakdown and lipolysis in the EDL. Similar effects of IL-6 on AMPK, energy state, and cAMP content were observed in C2C12 myotubes and gastrocnemius muscle in vivo, indicating that they were not unique to the incubated EDL.

CONCLUSIONS—These studies demonstrate that IL-6 activates AMPK in skeletal muscle by increasing the concentration of cAMP and, secondarily, the AMP:ATP ratio. They also suggest that substantial increases in IL-6 concentrations, such as those that can result from its synthesis by muscles during exercise, may play a role in the mobilization of fuel stores within skeletal muscle as an added means of restoring energy balance.

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Proliflammatory effects of interleukin-6 (IL-6) have been well documented (rev. in 1). In light of this and the presence of elevated plasma levels (two- to threefold) of IL-6 in patients with obesity, diabetes, and atherosclerotic cardiovascular disease (2,3), IL-6 has previously been viewed as a contributor to the development of these and other disorders associated with insulin resistance and the metabolic syndrome. However, studies in humans have demonstrated that IL-6 may have other functions. IL-6 is synthesized and released in large quantities from contracting skeletal muscle, resulting in plasma concentrations 50- to 100-fold higher than those seen at rest (rev. in 4). Furthermore, when IL-6 was infused into humans to achieve plasma levels similar to those seen with intense sustained exercise, it stimulated both lipolysis and fat oxidation (5), and no evidence of insulin resistance was observed. Cumulatively, these findings have led to the suggestion that muscle-derived IL-6 plays a role in regulating mammalian fuel homeostasis during exercise.

AMP-activated protein kinase (AMPK) is a fuel sensing enzyme that responds to cellular energy deficits by increasing catabolic processes that generate ATP (e.g., fatty acid oxidation) and downregulating anabolic processes that consume ATP but are not acutely required for cell survival (e.g., protein, glycerolipid, and cholesterol synthesis) (rev. in 6). The current view is that the primary mechanism by which AMPK is activated is through alterations in cellular energy state, as manifested by increases in the AMP:ATP ratio (rev. in 7). Hormones such as adrenaline (8), leptin (9), and ciliary neurotrophic factor (CNTF) (10) and pharmacological agents such as isoproterenol (11), thiazolidenediones (12), and metformin (13) have been reported to alter cellular energy state and activate AMPK in various tissues.

In addition to hormones and pharmacological agents, a number of studies have demonstrated that exercise also induces AMPK activity in multiple tissues (14). Previously, we have demonstrated that exercise increases AMPK activity in liver and adipose tissue, as well as muscle, in the rat leading to the notion that a systemic factor may be involved. In turn, the finding that increases in AMPK during exercise are markedly attenuated in these tissues in IL-6−/− mice suggested that one such factor could be IL-6 (15). The concurrent demonstrations that IL-6 can directly activate AMPK in skeletal muscle and adipose tissue, both in vivo and in vitro, also supported this notion (15,16). Despite these observations, the mechanism by which IL-6 activates AMPK in various tissues is not known. In the present study, we examined this question in skeletal muscle. The results indicate that IL-6 activates AMPK in an incubated rat extensor digitorum longus (EDL) preparation by increasing the concentrations of cAMP and, secondarily, the AMP:ATP ratio. They also suggest that IL-6 concurrently stimulates lipolysis, glycogenolysis, and fatty acid oxidation in this tissue.

RESEARCH DESIGN AND METHODS

Antibodies for P-AMPK (Thr172), P-STAT3 (Tyr705) cAMP-responsive element binding protein (P-CREB; Ser133), and phosphorylated glycogen synthase...
IL-6 ACTIVATES AMPK VIA cAMP

FIG. 1. Incubation with IL-6 increases α2 AMPK activity, protein phosphorylation, and UCP3 and PGC1α protein abundance in incubated EDL muscle. A: α2 AMPK activity (bar graph) and AMP/ATP ratio (line graph) were assayed in rat EDL muscles incubated with IL-6 (120 ng/ml) for 15–60 min. B: Dose-curve of α2 AMPK activity in response to IL-6 (15–120 ng/ml) for 30 min. C: Immunoblots of P-ACC (Ser79), P-STAT3 (Tyr705), P-AMPK (Thr172), P-CREB (Ser133), and β-actin in EDL muscles incubated with IL-6 (120 ng/ml) for 15 min. D: Immunoblots of PGC1α, UCP3, and β-actin in EDL incubated with IL-6 (120 ng/ml) for 30–120 min. E: Densitometric analysis of immunoblots of UCP3 and PGC1α protein shown in D. Results are means ± SE, n = 4–10. EDL muscles. *P < 0.05 versus control. ‡P < 0.05 versus 15 min IL-6. Immunoblots shown are representative of four samples.

In vitro experiments. Male Sprague-Dawley rats weighing 100 g (±5 g) were used for experiments in which they were injected with IL-6 (25 ng/g animal weight i.p.). Sixty minutes after the injection, animals were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and whole gastrocnemius muscle was excised, frozen in liquid nitrogen, and stored at −80°C until further analysis.

Cell culture experiments. C2C12 myoblasts were purchased from American Type Culture Collection (Rockville, MD). The cells were plated in six-well plates and cultured in DMEM containing 1% GlutMAX, 1% penicillin/streptomycin, and 10% FBS. When myoblasts reached 80% confluence, their differentiation to myotubes was induced by switching the media to DMEM containing 2% horse serum, 1% GlutMAX, and 1% penicillin/streptomycin. 

The authors used a series of experiments to study the effects of IL-6 on AMPK activity and protein phosphorylation in rat EDL muscles. They found that incubation with IL-6 increased α2 AMPK activity and AMP/ATP ratio. They also observed dose-curve of α2 AMPK activity in response to IL-6. In addition, they performed immunoblots of P-ACC (Ser79), P-STAT3 (Tyr705), P-AMPK (Thr172), P-CREB (Ser133), and β-actin in EDL muscles incubated with IL-6. They observed changes in protein abundance of UCP3 and PGC1α. Overall, their findings suggest that IL-6 activates AMPK via cAMP and may have implications for muscle biology and metabolism.
TABLE 1
IL-6 alters intracellular concentrations of adenine nucleotides and creatine phosphate

| ATP (µmol/g tissue) | ADP (µmol/g tissue) | AMP (µmol/g tissue) | Creatine phosphate (µmol/g tissue) |
|----------------------|----------------------|----------------------|----------------------------------|
| Control  | 10 | 15 | 30 | 60 |
| ATP  | 4.4 ± 0.2 | 3.8 ± 0.3 | 3.2 ± 0.3* | 4.1 ± 0.4 | 4.1 ± 0.2† |
| ADP  | 0.67 ± 0.02 | 0.84 ± 0.04 | 0.73 ± 0.12 | 0.67 ± 0.04 | 0.83 ± 0.11 |
| AMP  | 0.04 ± 0.004 | 0.045 ± 0.003 | 0.07 ± 0.006* | 0.05 ± 0.007 | 0.03 ± 0.007† |
| Creatine phosphate | 13.9 ± 0.5 | 10.9 ± 0.5* | 10.7 ± 0.8* | 12.5 ± 1.0 | 12.9 ± 0.4† |

Data are means ± SE (n = 5-8). Rat EDL muscles were incubated with IL-6 (120 ng/ml) for 10–60 min. Because no significant differences in the nucleotide content were observed in the control muscles at the different time points, values for control muscles at all time points were pooled for statistical purposes. *P < 0.05 vs. control. †P < 0.05 vs. 15 min IL-6.

AMPK activity. AMPK activity was assayed in frozen skeletal muscle homogenized in lysis buffer as described previously (18). Briefly, muscle lysate containing 200 µg protein was immunoprecipitated with antibody specific to the α2 or α1 catalytic subunit of AMPK and protein A/G agarose beads. Beads were washed five times, and the activity of the immobilized enzyme was assayed based on the phosphorylation of “SAMS” peptide (0.2 µmol/l) by 0.2 µmol/l ATP (containing 2 µCi [γ-32P]ATP) in the presence and absence of 0.2 µmol/l AMP. Label incorporation into the SAMS peptide was measured on a Racheta 1254 scintillation counter.

Western blotting. Cultures were scraped on ice in cell lysis buffer (Cell Signaling Technology, Beverly, MA) (plus 1 µmol/l phenylmethylsulfonyl fluoride and 1:2,000 for P-ACC, T-GSK3, and 1:1,000 for P-AMPK, P-STAT3 and phospho-Tyr 210 [Tyrosine] (for P-AMPK at Thr172) and Thr173 (for P-ACC); 1:1,000 for P-ACC, T-GSK3, and T-P-ACC; 1:2,000 for P-AMPK, P-ACC, T-GSK3, and T-P-ACC; 1:1,000 for P-STAT3; 1:1,000 for P-PGClα; and 1:500 for P-CREB and UCPI3) and then in TBST containing 5% nonfat dry milk and the appropriate secondary antibody conjugated to horseradish peroxidase at a 1:5,000 dilution. After this, the membranes were subjected to an enhanced chemiluminescence reaction for 1 hour. Blots were then washed five times, and the activity of the immobilized enzyme was assayed based on the phosphorylation of “SAMS” peptide (0.2 µmol/l) by 0.2 µmol/l ATP (containing 2 µCi [γ-32P]ATP) in the presence and absence of 0.2 µmol/l AMP. Label incorporation into the SAMS peptide was measured on a Racheta 1254 scintillation counter.

Other measurements. Lactate release into the incubation medium was determined spectrophotometrically as described previously (20), and glycerol release was determined with a commercially available kit after concentration of the medium by lyophilization (Sigma). Tissue glycogen content was determined using the phenol-sulfuric acid reaction (21). Statistical analyses. Results are expressed as means ± SE. Statistical significance was determined by one-way or two-way ANOVA for nonrepeated measures, as appropriate. The Bonferroni post hoc test was used in the event of a significant (P < 0.05) ratio.

RESULTS
IL-6 activates α2 AMPK in intact rat skeletal muscle. The time course and dose response of AMPK activation by IL-6 are presented in Fig. 1A and B. Incubation of EDL muscle with IL-6 (120 ng/ml) has been shown previously to induce a twofold increase in the phosphorylation of the α subunit of AMPK at Thr172 (an index of its activity) after 15 min, with values returning to baseline by 60 min (15). As shown in Fig. 1A, an almost identical pattern was observed when the activity of the immunoprecipitated α2 isoform of AMPK was measured. In contrast, no change in the activity of the α1 isoform of AMPK was observed (data not shown). Activation of the α2 AMPK occurred at IL-6

**FIG. 2.** IL-6–induced increases in α2 AMPK activity, cAMP levels, and AMP:ATP ratio are abrogated by propranolol (Propr). A: Activity of α2 AMPK (bar graph) and AMP:ATP ratio (line graph) in EDL pretreated or not pretreated for 30 min with the β-adrenergic antagonist propranolol (10 µmol/l) and then with or without IL-6 (120 ng/ml) for 15 min. B: cAMP levels in EDL muscles incubated with IL-6 (120 ng/ml) for 15 or 30 min. C: cAMP levels in EDL preincubated with or without propranolol (10 µmol/l) for 30 min and then with or without IL-6 (120 ng/ml) for 15 min. Results are means ± SE (n = 4–7). *P < 0.05 versus control, no propranolol; †P < 0.05 versus IL-6 no propranolol; ‡P < 0.05 versus 15-min time point.
concentrations as low as 15 ng/ml; however, it was somewhat less than that observed at 120 ng/ml (Fig. 1B). Incubation with IL-6 (120 ng/ml) for 15 min increased the phosphorylation of AMPK (Thr 172) and of its downstream target ACC (Ser79) (Fig. 1C) in keeping with the changes in AMPK activity. Thereafter, P-AMPK and P-ACC returned to baseline values after 60 min and remained so for up to 4 h, the maximum length of incubation tested (data not shown). IL-6 also increased the phosphorylation of STAT3 (Tyr 705), a known event in the activation of the JAK/STAT signaling cascade (22) (Fig. 1C). Finally, although the increase in AMPK activity was transient, it was followed by two- and fourfold increases in UCP3 protein abundance after 1 and 2 h, respectively (Fig. 1D and E). A smaller increase in PGC1α protein expression (25%) was observed; however, it only achieved statistical significance after 2 h (Fig. 1D and E).

**IL-6 alters the levels of intracellular adenine nucleotides.** As a first step in studying the mechanism by which IL-6 activates AMPK, we examined its effect on cellular energy state. Control muscles gave similar values to those reported previously (23); however, incubation with IL-6 (120 ng/ml) led to decreases in the concentrations of creatine phosphate and ATP, no significant changes in ADP, and an increase in AMP levels (Table 1). This resulted in increases in the AMP:ATP ratio that paralleled those of AMPK activity, with both of them being maximal at 15 min and returning to baseline by 60 min (Fig. 1A).

**The effects of IL-6 on AMPK activity are dependent on cAMP production and energy state alterations.** Koh et al. (8) have reported that the injection of adrenaline activates AMPK and increases the AMP:ATP ratio in rat adipose tissue and that these changes are inhibited by the β-adrenergic antagonist propranolol. Here we found that propranolol, at a concentration of 10 μmol/l, also inhibited IL-6–induced AMPK activation, raising the possibility that

![Graph](image.png)

**FIG. 3.** The adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine (ddA) blocks IL-6-induced α2 AMPK activity. Activity of α2 AMPK in EDL pretreated (□) or not pretreated (□) with dideoxyadenosine (100 μmol/l) for 30 min and then with or without IL-6 (120 ng/ml) for 15 min. Results are means ± SE (n = 4–6), *P < 0.05 versus control, no inhibitor, †P < 0.05 versus IL-6 no inhibitor.

![Chart](chart.png)

**FIG. 4.** IL-6 exerts similar effects in C2C12 myotubes and gastrocnemius muscle in vivo. Immunoblots of P-ACC (Ser79), P-STAT3 (Tyr705), P-AMPK (Thr172), P-CREB (Ser133), and β-actin were performed on (A) lysates of C2C12 myotubes treated with IL-6 (50 ng/ml) or isoproterenol (iso) (10 μmol/l) for 30 min and (B) homogenates of gastrocnemius muscle of rats injected intraperitoneally with saline (veh) or 2.5 mg of IL-6 1 h before being killed. Immunoblots shown are representative of n = 3. C: cAMP levels assayed in extracts of C2C12 myotubes treated for 15 min with IL-6 (50 ng/ml). D: Adenine nucleotide and cAMP levels in muscles studied in B. Results are means ± SE, n = 3–4. *P < 0.05 versus control.
IL-6 caused β-adrenergic stimulation (Fig. 2A). In keeping with this notion, 15-min incubation with IL-6 (120 ng/ml) induced an increase in both cAMP levels and the phosphorylation of its downstream target P-CREB at Ser133 (Figs. 2B and 1C). As expected, the IL-6–induced increase in cAMP was prevented by propranolol (Fig. 2C). In addition, propranolol prevented the increases in the AMP:ATP ratio caused by IL-6 (Fig. 2D). As shown in Fig. 1A, the alterations in the AMP:ATP ratio (Fig. 2A, line-graph) coincided with changes in α2 AMPK activity (Fig. 2A, bar graph). Finally, consistent with these results, activation of AMPK by IL-6 was also prevented by the adenyl cyclase inhibitor 2′,5′-dideoxyadenosine (Fig. 3).

The effects of IL-6 on energy state and cAMP are also observed in cultured C2C12 myotubes and gastrocnemius muscle in vivo. Treatment of C2C12 cells with IL-6 increased cAMP, P-ACC, P-AMPK, P-STAT3, and P-CREB abundance, as did isoproterenol (Fig. 4A and C), suggesting both that the effects of IL-6 on the EDL are not unique to the incubated muscle and that they can be mimicked by isoproterenol, a known β-adrenergic agonist. In keeping with the former conclusion, IL-6 injected intraperitoneally for 60 min also led to increases in the abundance of P-ACC, P-AMPK, P-STAT3, and P-CREB and in the AMP:ATP ratio in the gastrocnemius muscle, just as did it in the incubated muscle (Fig. 4B and D).

**IL-6 increases lipolysis and glycogenolysis.** IL-6 has been reported to increase whole-body lipolysis (5) and decrease glycogen content in primary hepatocytes (24), as do catecholamines, glucagon, and other agents that increase cAMP. For this reason, we assessed whether IL-6 had similar effects on muscle. As shown in Fig. 5A, incubation of the EDL with IL-6 (120 ng/ml) for 120 min diminished muscle glycogen by 30%. In keeping with this, IL-6 treatment decreased the phosphorylation of GSK3β, suggesting that it inhibited glycogen synthase activity (Fig. 5B). (Phosphorylase A and glycogen synthase activity per se were not assayed.) The decrease in GSK3β phosphorylation was not affected by propranolol, indicating that it is independent of AMPK activation. IL-6 also caused a 1.5-fold increase in lactate release into the media and increased the release of glycerol, suggesting that glycolysis and lipolysis were also stimulated by this treatment (Fig. 5C and D). Finally, in agreement with previous studies in cultured myotubes and soleus muscle (25), incubation of the EDL for 30 min with IL-6 caused a fivefold increase in...
palmitate oxidation (Fig. 5E), an effect attributed to AMPK activation.

DISCUSSION

IL-6 acutely activates AMPK in skeletal muscle both in vivo and in vitro (15,16,25,26). The results of the present study indicate that it does so by altering events that increase intracellular cAMP and, secondarily, the AMP:ATP ratio. In support of this notion, IL-6–induced AMPK activation in the incubated EDL was accompanied by increases in cAMP, and this, as well as the increase in the AMP:ATP ratio, was completely inhibited by the β-adrenergic antagonist propranolol. Additionally, the adenylyl cyclase inhibitor 2’5’ dideoxyadenosine blocked the activation of AMPK by IL-6. Finally, IL-6 caused similar changes in C2C12 myotubes and gastrocnemius muscle in vivo, indicating its effects were not unique to the incubated EDL.

How the IL-6–induced elevation of cAMP in turn increased the AMP:ATP ratio remains to be determined. Another agent that increases cAMP in skeletal muscle, isoproterenol, also has been reported to increase AMPK activity (27), lipolysis, and glycogenolysis (28); however, its effect on energy state was not examined. On the other hand, such measurements have been carried out in adipose tissue. Investigations in 3T3-L1 adipocytes have demonstrated that increases in the AMP:ATP ratio and AMPK activation occur when cAMP is increased by either forskolin or isoproterenol (11) and that they are dependent on the stimulation of lipolysis. Studies by Koh et al. (8) have suggested that a similar mechanism occurs in rat adipose tissue in vivo, in that exercise and adrenaline (epinephrine)-induced increases in the AMP:ATP ratio and AMPK activity were both inhibited by propranolol. Recently, it has been reported that IL-6 induces lipolysis in porcine adipocytes and that this effect appears to be dependent on the actions of IL-6 on ERK1/2 and its ability to directly phosphorylate hormone-sensitive lipase (HSL) (29); however, the role of AMPK in this setting was not examined. Whether the stimulation of lipolysis accounts for the IL-6–induced changes in energy state and AMPK activity in the EDL muscle as it does in adipose tissue remains to be determined.

It has been clearly demonstrated that cAMP and protein kinase A (PKA) activation increase the biosynthesis of IL-6 in various cells (30); however, IL-6–induced increases in cAMP have not been previously reported. Thus, one major question is, how does IL-6 induce cAMP production? We speculate that an intermediary molecule could be protein kinase C-8 (PKC8), given that this enzyme mediates the activation of the cAMP producing adenylyl cyclase isoform VII (31) and that IL-6 induces its membrane translocation in hepatocytes (32). Also, PKC8 mediates the binding of the IL-6 transmembrane receptor gp130 to STAT3 (33,34), and it has been implicated in IL-6–induced insulin signaling (34).

The effects of IL-6 on the phosphorylation of CREB (Ser133) have not been reported previously; however, it has been noted that both AMPK and PKA phosphorylate CREB at the same residue (Ser133) (35). In addition, both AMPK (36,37) and CREB (38) appear to regulate the abundance of UCP3 and PGC1α. Whether AMPK or PKA is responsible for the IL-6–induced increases in UCP3 and PGC1α observed in the present study remains to be determined experimentally. It is noteworthy that these effects of IL-6 are qualitatively similar to those produced by leptin and CNTF (10,39), both of which also induce a rapid and transient activation of AMPK, increase the AMP:ATP ratio (9,10), and activate the JAK/STAT signaling cascade (22). Recently, it has been reported that STAT3 can localize in mitochondria and enhance oxidative phosphorylation (40), suggesting that it could directly influence cellular ATP levels.

In previous reports, a concentration of IL-6 (120 ng/ml) well above that in plasma was used to induce AMPK activation in incubated rat skeletal muscle (15,26,41). In the present study, AMPK activation was observed at IL-6 concentrations as low as 15 ng/ml; however, this is still significantly higher than the reported resting concentration of IL-6 in the plasma of a rat (10–300 pg/ml) (42,43). During sustained exercise, the muscle cell is most likely exposed to much higher concentrations of IL-6 given that it is synthesizing IL-6 and releasing it into the circulation at amounts sufficient to increase its plasma concentration dramatically. Thus, in humans, IL-6 release from muscle during exercise can result in plasma concentrations 50- to 100-fold higher than those at rest (44) and concentrations

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**FIG. 6.** Paracrine functions of IL-6 synthesized and released by muscle during exercise. During sustained intense exercise, IL-6 is synthesized and released from skeletal muscle cells and increases muscle cAMP, AMP:ATP ratio, and AMPK activity. This results in enhanced muscle fatty acid oxidation, lipolysis, and glycogenolysis.
in the interstitial fluid of muscle 10- to 100-fold higher than those in plasma (45,46). In one study, an interstitial fluid IL-6 concentration of 1.2 ng/ml was observed in humans after low-grade repetitive exercise without a detectible change in plasma IL-6 (46). Therefore, in the rat, if one assumes a low (~10 pg/ml) resting plasma IL-6 level and an effect of exercise similar to that in humans, an interstitial fluid IL-6 concentration between 10–100 ng/ml would be achieved during intense exercise; this, however, remains to be determined.

It has been proposed that IL-6 synthesized and released into the circulation by skeletal muscle during exercise is an endocrine signal from the muscle cell that is becoming fuel deficient (4). In keeping with this notion, IL-6 increases adipose tissue lipolysis and hepatic glycogenolysis (during exercise) in humans (5,47) and its release from muscle is enhanced when muscle glycogen is depleted (48). As already noted, large increases in muscle interstitial fluid IL-6 have been observed in humans after low-intensity exercise in the absence of a change in plasma IL-6. This, together with the results of the present study, suggest that IL-6 could also act as an autocrine or paracrine factor that enhances lipolysis and glycogenolysis and activates AMPK in the muscle cell during exercise (Fig. 6).

Finally, the physiological relevance of IL-6 in this setting is strongly suggested by the observation that both the ability to sustain exercise and activate AMPK are impaired in IL-6−/− mice (15,49,50).

In summary, the results indicate that IL-6, at concentrations hypothetically representative of those released during exercise, activates AMPK in skeletal muscle by increasing cAMP production and, secondarily, the AMP:ATP ratio. They also indicate that this transient activation of AMPK results in a more sustained increase in the protein expression of two AMPK-mediated genes that act on mitochondria, PGC1α, and UCP3. Finally, they reveal that IL-6 increases substrate availability within the muscle cell by increasing glycogenolysis and lipolysis. Studies in IL-6−/− mice have established that a lack of IL-6 markedly inhibits AMPK activation during exercise (15), whether it also diminishes the increases in cAMP levels, and PGC1α and UCP3 expression caused by exercise, remains to be determined.

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