β-Adrenergic stimulation does not activate p38 MAP kinase or induce PGC-1α in skeletal muscle

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Kim SH, Asaka M, Higashida K, Takahashi Y, Holloszy JO, Han D. β-Adrenergic stimulation does not activate p38 MAP kinase or induce PGC-1α in skeletal muscle. Am J Physiol Endocrinol Metab 304: E844–E852, 2013. First published March 5, 2013; doi:10.1152/ajpendo.00581.2012—There are reports that the β-adrenergic agonist clenbuterol induces a large increase in peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) in skeletal muscle. This has led to the hypothesis that the increases in PGC-1α and mitochondrial biogenesis induced in muscle by endurance exercise are mediated by catecholamines. In the present study, we evaluated this possibility and found that injecting rats with clenbuterol or norepinephrine induced large increases in PGC-1α and mitochondrial proteins in brown adipose tissue but had no effect on PGC-1α expression or mitochondrial biogenesis in skeletal muscle. In brown adipocytes, the increase in PGC-1α expression induced by β-adrenergic stimulation is mediated by activation of p38 mitogen-activated protein kinase (p38 MAPK), which phosphorylates and activates the cAMP response element binding protein (CREB) family member activating transcription factor 2 (ATF2), which binds to a cyclic AMP response element (CRE) in the PGC-1α promoter and mediates the increase in PGC-1α transcription. Phospho-CREB does not have this effect. Our results show that the reason for the lack of effect of β-adrenergic stimulation on PGC-1α expression in muscle is that catecholamines do not activate p38 or increase ATF2 phosphorylation in muscle.

phospho-ATF2; clenbuterol; norepinephrine; mitochondria; brown fat

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-γ COACTIVATOR 1α (PGC-1α) was discovered during a study of the regulation of uncoupling protein-1 (UCP1) expression by the nuclear receptor PPARγ during induction of adaptive thermogenesis in brown fat (34). PGC-1α expression is powerfully induced in brown fat cells of mice exposed to cold (34). This effect is mediated by the cold-induced increase in catecholamine secretion and is mimicked by treatment with β2-adrenergic agonists (34). Skeletal muscle also undergoes an increase in PGC-1α in cold-exposed mice (34). Subsequent studies led to the discovery that PGC-1α is a transcription coactivator that activates and coordinates transcription of mitochondrial enzymes and that increased expression of PGC-1α results in increased mitochondrial biogenesis (18, 37, 47). It has been known for many years that endurance exercise results in an increase in mitochondria in skeletal muscle (21, 22). This increase in mitochondria, which is limited to the muscles performing the exercise, can be as great as two- or threefold if the exercise is sufficiently intense and prolonged (20, 23). It plays a key role in the training-mediated increase in exercise capacity (9, 20). For a long time, nothing was known regarding the mechanism by which the increase in mitochondria is mediated. After the discovery of PGC-1α, exercise was shown to result in both activation and increased expression of PGC-1α (5, 15, 46).

In studies of the mechanism by which exercise induces an increase in PGC-1α, Ezaki and coworkers (27, 28, 40) injected rats with the β2-adrenergic agonist clenbuterol. They reported a large increase in PGC-1α mRNA in skeletal muscle 4 h after injection. They also reported that β-adrenergic blockade with propranolol prevented an exercise-induced increase in PGC-1α (27, 28, 40) and concluded that the effect of exercise on PGC-1α expression is mediated by the increase in catecholamines that occurs during exercise. Similarly, Chinsomboon et al. (8) reported that PGC-1α mRNA was increased in muscle 6 h after clenbuterol injection and that, in addition, PGC-1α protein expression was strongly induced at the same time. There is a cyclic AMP response element (CRE) site in the PGC-1α promoter (17) that plays an essential role in the exercise-induced increase in muscle mitochondria (1, 3), and these investigators (8, 27, 28, 40) hypothesized that the increase in PGC-1α was mediated by the phosphorylation of the cyclic AMP response element binding protein (CREB) induced by the β-adrenergic stimulus. Another explanation was provided by Gerhart-Hines et al. (14) and by Park et al. (31), who implicated silent mating type information regulator 2 homolog 1 (SIRT1). Gerhart-Hines et al. (14) reported that, in muscles of mice injected with clenbuterol, and in cells treated with β-adrenergic agonists, SIRT1 was phosphorylated and activated, leading to PGC-1α deacetylation, activation, and increased expression. Park et al. (31) reported that inhibition of cAMP-degrading phosphodiesterases leads to an elevation of cAMP, which, by a complicated series of reactions, results in activation of SIRT1 and an increase in PGC-1α.

That β-adrenergic stimulation could result in increased expression of PGC-1α in muscle seems plausible in light of the increases in PGC-1α and mitochondrial biogenesis induced in brown adipocytes by β-adrenergic stimulation and of the increase in mitochondrial biogenesis in muscles of cold-exposed mice (34). However, we found these reports puzzling for the following reasons. In a study to evaluate the possibility that β-adrenergic stimulation might mediate the exercise-induced increase in mitochondrial biogenesis, an experiment was performed in 1981 in which rats were injected with large doses of epinephrine daily for 6 wk; the epinephrine treatment had no effect on muscle content of mitochondria (12). Clenbuterol, the β2-adrenergic agonist used in the studies reviewed above, is a potent anabolic agent used to stimulate muscle growth in body builders and in animals raised for meat (29, 32, 38). It is well documented that clenbuterol causes a decrease, not an increase, in muscle mitochondria, with a shift from oxidative to glycolytic muscle fibers (24, 29, 32, 39, 48).
Spiegelman’s group found that, while bromo-cAMP induces an increase in PGC-1α expression in brown adipocytes, it does not in C2C12 myotubes (47).

In this context, the purpose of our study was to determine whether clenbuterol/β-adrenergic stimulation induces an increase in expression of PGC-1α in muscle and, if not, to determine why not. Our results show that β-adrenergic stimulation does not induce an increase in PGC-1α or mitochondrial biogenesis in muscle. The explanation is that the CRE in the PGC-1α promoter that regulates PGC-1α expression is activated by phosphorylated activating transcription factor 2 (phospho-ATF2), not by phospho-CREB (2, 7). ATF2, which is a CREB family member, is phosphorylated by p38 mitogen-activated protein kinase (p38 MAPK, p38), which is activated by β2-adrenergic stimulation in brown adipose tissue (7) but not in muscle.

MATERIALS AND METHODS

Animals and β-adrenergic agonist injections. This research was approved by the Animal Studies Committee of Washington University School of Medicine. Male Wistar rats weighing ~100 g were obtained from Charles River Laboratories (Wilmington, MA) and individually housed in a temperature- and light-controlled animal facility. They were fed a rodent laboratory chow diet (Purina, St. Louis, MO) and provided with water ad libitum.

Rats were injected subcutaneously with 1 mg/kg body wt clenbuterol or 1 mg/kg body wt norepinephrine, and triceps and gastrocnemius muscles and brown adipose tissue were harvested at various time intervals after injection. Rats were anesthetized by injection with pentobarbital sodium, and, after the muscles and brown adipose tissue were dissected out, the rats were euthanized by exsanguination. Tissues were kept at −80°C until analyzed.

Exercise protocol. Rats were exercised by swimming as described previously (5), except that the duration of the swim was 30 min.

C2C12 muscle cell culture and treatments. C2C12 mouse myoblasts were grown in DMEM (4.5 g/l glucose, Invitrogen) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Differentiation was initiated by switching to medium containing 2% heat-inactivated horse serum when myoblasts were 90% confluent. After 48 h of differentiation, batches of myotubes were treated with 1 μM clenbuterol or 0.1 μM anisomycin for the time periods shown in the figure legends.

Fig. 1. Effects of β-adrenergic stimulation on peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) transcription. A: clenbuterol treatment has no effect on PGC-1α mRNA level in skeletal muscle 6 h after injection. B: norepinephrine treatment has no effect on PGC-1α mRNA level in skeletal muscle 6 h after injection. C: PGC-1α mRNA is increased in brown adipose tissue 6 h after clenbuterol injection. D: PGC-1α mRNA is increased in brown adipose tissue 6 h after norepinephrine injection. Values are means ± SE; n = 12 rats per group. *P < 0.01 vs. saline.
PGC-1α promoter activity assay. To evaluate the effects of the β-adrenergic receptor agonists, and of p38 MAPK activation with anisomycin, on PGC-1α promoter activity, C2C12 myoblasts that had attained 70% confluence were cotransfected with 600 ng of PGL3-PGC-1α reporter (Promega) and 50 ng of CMV promoter-driven Renilla luciferase (pRL-CMV, Promega) and either 600 ng of DN-ATF2 (Addgene plasmid 33362) or empty plasmid (Addgene plasmid 33348), using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. Thirty

hours after transfection, luciferase was measured using a Dual-Glo luciferase assay system (Promega) according to the manufacturer’s instructions. Transfection efficiencies were normalized with Renilla luciferase activity.

Western blot analysis. Frozen tissues were homogenized (16), and Western blots were prepared (4) as described previously. The blots were probed with the following antibodies: PGC-1α (cat. no. 5165517 Calbiochem), cytochrome oxidase subunit IV (COX IV; cat. no. A21348, Fig. 2. Effects of β-adrenergic stimulation on PGC-1α expression in muscle and brown adipose tissue and on mitochondrial enzymes in muscle. A: clenbuterol treatment has no effect on PGC-1α protein expression measured in skeletal muscle 6 or 18 h after injection. B: noradrenaline treatment has no effect on PGC-1α protein expression in muscle 6 or 18 h after injection. C: clenbuterol has no effect on expression of mitochondrial respiratory chain proteins in muscle 18 h after injection. D: noradrenaline has no effect on expression of mitochondrial respiratory chain proteins in muscle 18 h after injection. E: clenbuterol induces increases in PGC-1α protein in brown adipose tissue measured 6 and 18 h after injection. F: noradrenaline induced increases in PGC-1α protein in brown adipose tissue measured 6 and 18 h after injection. Values are means ± SE; n = 6–10 rats per group. *P < 0.05 vs. saline.
CLENBUTEROL DOES NOT INDUCE PGC-1α IN MUSCLE

Invitrogen), succinate-ubiquinone oxidoreductase (SUO; cat. no. 459230, Invitrogen), NADH ubiquinone oxidoreductase (NADH-UO; cat. no. 459100, Invitrogen), ubiquinone-cytochrome c oxidoreductase Core 2 subunit (Core 2; cat. no. 459220, Invitrogen), cytochrome c (Cyto c; cat. no. 556433, BD Bioscience), β-actin (cat. no. A5441, Sigma), p38 MAPK (cat. no. 9212, Cell Signaling), phospho-p38 MAPK (cat. no. 9216, Cell Signaling), ATF2 (cat. no. 9226, Cell Signaling), CREB (cat. no. 9197, Cell Signaling), p-CREB (cat. no. 9212, Cell Signaling), phospho-p38 MAPK (cat. no. 9212, Cell Signaling), and p-ATF2 (cat. no. 9225, Cell Signaling). Antibody-bound protein was detected using enhanced chemiluminescence and quantified by densitometry.

**Determination of mRNA.** PGC-1α mRNA was determined using semiquantitative RT-PCR as described previously (41), using the primers forward 5′-GTGCCAGGCAAGACTCTGATGG-3′ and reverse 5′-GTCCAGGTCTATTACATCAAGTTC-3′. Transcript intensity was expressed relative to 18S (Ambion, Austin, TX).

**Statistics.** Values are expressed as means ± SE. Statistically significant differences were determined using unpaired Student’s t-test or ANOVA for multiple comparisons.

**RESULTS**

PGC-1α mRNA does not increase in skeletal muscle in response to β-adrenergic stimulation. There was no increase in PGC-1α mRNA in triceps muscle 6 h after clenbuterol injection (Fig. 1A). Norepinephrine injection also did not result in an increase in PGC-1α mRNA measured 6 h after injection (Fig. 1B). In contrast, there was a large increase in PGC-1α mRNA in brown adipose tissue 6 h following clenbuterol or norepinephrine injection (Fig. 1, C and D).

PGC-1α protein does not increase in muscle in response to β-adrenergic stimulation. There were no increases in PGC-1α protein expression in skeletal muscle either 6 h or 18 h after injection of either clenbuterol or norepinephrine (Fig. 2, A and B). There were also no increases in a number of mitochondrial enzyme proteins 18 h after injection with either clenbuterol or norepinephrine (Fig. 2, C and D). This is in contrast to the increases in PGC-1α protein in brown adipose tissue measured 6 h and 18 h following β-adrenergic stimulation (Fig. 2, E and F).

β-Blockade does not prevent the increase in PGC-1α mRNA induced by AICAR injection. The research groups of Ezaki (28, 40) and Arany (8) have reported that β-blockade prevents the increase in PGC-1α mRNA induced by exercise. We are not able to exercise β-blocked, untrained rats vigorously and have, therefore, not evaluated the effect of β-blockade on the response of PGC-1α to exercise. However, Ezaki’s group has also reported that injection of the “exercise mimetic” AICAR, which activates AMPK, results in an increase in catecholamines and that most of the AICAR-induced increase in PGC-1α mRNA is prevented by β-blockade (40). As shown in Fig. 3, we found that injection of rats with the β-blocker propranolol (10 mg/kg body wt) did not prevent the increase in muscle PGC-1α mRNA induced in gastrocnemius muscle by AICAR injection. Actually, the increase in PGC-1α mRNA induced by AICAR was greater in the β-blocked animals. We do not have an explanation for this greater increase.

**Effects of β-adrenergic stimulation on CREB, ATF2, and p38 phosphorylation.** There is a CRE in the PGC-1α promoter (17) that is essential for the adaptive increases in PGC-1α in brown adipose tissue in response to β-adrenergic stimulation (7) and in skeletal muscle in response to exercise (1). It has been reported that this CRE is activated by the CREB family member phospho-ATF2, not by phospho-CREB (1, 2, 7). As shown in Fig. 4, clenbuterol treatment induced an increase in CREB phosphorylation (Fig. 4A) but not in ATF2 phosphorylation (Fig. 4B) in skeletal muscle. In both muscle (2, 46) and brown adipocytes (7), ATF2 is phosphorylated by p38 MAPK. β-Adrenergic stimulation does not result in phosphorylation/activation of p38 MAPK in skeletal muscle (Fig. 4C), which explains why ATF2 is not phosphorylated. In contrast, β-adrenergic stimulation activates p38 MAPK in brown adipocytes (7) (Fig. 5A), resulting in an increase in ATF2 phosphorylation (Fig. 5B). PGC-1α promoter activity was measured in C2C12 myotubes that had been transfected with a PGC-1α promoter-luciferase construct. The PGC-1α promoter was not activated in response to β-adrenergic stimulation in myotubes (Fig. 4D).

**Activation of p38 MAPK in C2C12 myotubes and skeletal muscle.** Treatment of C2C12 myotubes with anisomycin, an activator of p38 MAPK (19) (Fig. 6A), induced an increase in ATF2 phosphorylation (Fig. 6B) but not in CREB phosphorylation (Fig. 6C). Activation of p38 MAPK with anisomycin resulted in increased PGC-1α promoter activity measured in C2C12 myotubes that had been transfected with a PGC-1α promoter-luciferase construct (Fig. 6D). The anisomycin-induced increase in PGC-1α promoter activity was blocked by expression of a dominant negative ATF2 in the myotubes (Fig. 6D). As in previous studies (2, 46), exercise, in contrast to β-adrenergic stimulation, resulted in p38 MAPK phosphorylation (Fig. 7A) and increased phosphorylation of ATF2 (Fig. 7B) in rat skeletal muscle.

**DISCUSSION**

Endurance exercise, such as running or swimming, induces an adaptive increase in skeletal muscle mitochondria with a shift in fiber type to red/high oxidative (20). There is evidence...
that the increase in cytosolic Ca\(^{2+}\) that mediates excitation-contraction coupling and the increase in AMP resulting from ATP breakdown during muscle contraction are involved in mediating the increase in mitochondrial biogenesis induced by exercise (20). The increase in Ca\(^{2+}\) results in activation of calcium/calmodulin-dependent protein kinase II (CAMKII), which is the first step in a pathway leading to activation of p38 MAPK (13, 30, 45). Activated p38 phosphorylates and activates PGC-1\(\alpha\) (11, 33). p38 also phosphorylates and activates ATF2, a transcription factor that regulates transcription of the gene encoding PGC-1\(\alpha\) (1, 2, 7). Thus, Ca\(^{2+}\) stimulates mitochondrial biogenesis both via activation of PGC-1\(\alpha\) and via increased expression of PGC-1\(\alpha\). AICAR, which is taken up by muscle and converted to the AMP analog ZMP, activates AMPK. Studies using AICAR have shown that AMPK activation induces an increase in muscle mitochondria (35, 44). AMPK phosphorylates and activates PGC-1\(\alpha\) (26), and it is therefore probable that AMPK activation also plays a role in the increase in muscle mitochondria induced by exercise (20).

Vigorous exercise results in large increases in epinephrine and norepinephrine levels (10, 43), and the possibility that this increase in catecholamines might be involved in mediating the exercise-induced increase in mitochondria was investigated long before the discovery of PGC-1\(\alpha\) (12). It was found that daily injection of a large dose of epinephrine for 6 wk had no effect on the levels of three mitochondrial enzymes or in the capacity of skeletal muscle to oxidize pyruvate (12). It was concluded that catecholamines are not responsible for inducing the increase in muscle mitochondria during exercise. These results were not surprising, as the adaptations to exercise are limited to exercised muscle, whereas all the muscles are exposed to the increased circulating levels of catecholamines.

In light of this information, we were surprised by the reports that \(\beta\)-adrenergic stimulation or raising cyclic AMP using
Our findings raise the question: why does β-adrenergic stimulation induce an increase in PGC-1α expression and in mitochondrial biogenesis in brown adipose tissue and not in skeletal muscle? There is a CRE in the promoter of the PGC-1α gene that is essential for the adaptive response of brown adipose tissue to cold and β-adrenergic stimulation (7) and for the increases in PGC-1α and mitochondrial biogenesis in response to contractile activity in skeletal muscle (3). In adipose tissue, the CRE in the PGC-1α promoter is activated by the CREB family member phospho-ATF2, not phospho-CREB (7). The present results show that phospho-ATF2, not phospho-CREB, is also responsible for activating the CRE in the PGC-1α promoter in skeletal muscle. This finding is in keeping with the observation by Akimoto et al. (1) that a dominant negative form of ATF2 completely blocks the increase in PGC-1α promoter activity induced by exercise.

ATF2 is phosphorylated and activated by p38 MAPK (2, 7, 45, 46) in both skeletal muscle and brown adipose tissue. In brown adipocytes, p38 MAPK is activated by β-adrenergic stimulation (7); the mechanism involves activation of MAP kinase kinase 3 (36). In skeletal muscle, exercise results in activation of p38 MAPK (2, 25, 42, 46), apparently by a pathway activated by Ca2+ (45), resulting in phosphorylation of ATF2 (2, 45). As shown in the present study, β-adrenergic stimulation induces an increase in phospho-CREB but not in phospho-ATF2 in skeletal muscle because of an inability to activate p38 in muscle. This finding explains why β-adrenergic stimulation does not induce an increase in mitochondrial biogenesis in skeletal muscle.

The finding that β-adrenergic stimulation does not induce increases in PGC-1α expression or mitochondrial biogenesis in muscle raises the question: how does cold exposure induce increases in PGC-1α expression and mitochondrial biogenesis in muscle? Skeletal muscle is not capable of adaptive thermogenesis, because it does not contain UCP1. UCP2 and UCP3, which are expressed in muscle, have been termed uncoupling proteins because of some structural similarities to UCP1; however, they do not uncouple ATP synthesis from electron transport in mitochondria and cannot, therefore, mediate adaptive thermogenesis (6). Skeletal muscles adapt to cold with shivering thermogenesis, which involves muscle contractions and is a form of exercise.

The finding that β-adrenergic stimulation does not induce an increase in mitochondrial biogenesis in muscle has practical relevance to sports doping. Competitive athletes and their advisors search the scientific literature for potential performance enhancers and are by now probably aware of the reports that clenbuterol induces PGC-1α in muscle. That increasing muscle mitochondria improves endurance is well known, and β-adrenergic agents would be ideal doping substances, if they were effective, because of their very short half-lives and, therefore, the inability to detect them. Repeated large doses of catecholamines are potentially harmful. The observation that β-adrenergic stimulation has no effect on mitochondrial biogenesis should have the beneficial effect of discouraging their use as doping agents by endurance athletes.

In conclusion, our results show that clenbuterol/β-adrenergic stimulation does not induce increases in PGC-1α expression or mitochondrial biogenesis in muscle. The explanation for this lack of effect is that, in contrast to brown adipose tissue, β-adrenergic stimulation does not activate p38 MAPK or increase ATF2 phosphorylation in skeletal muscle.

Fig. 5. Clenbuterol activates p38 MAPK and ATF2 in brown adipose tissue. A: p38 MAPK phosphorylation is increased in brown adipose tissue 30 min after clenbuterol injection. B: ATF2 phosphorylation is increased in brown adipose tissue 30 min after clenbuterol injection. Values are means ± SE; n = 8 rats per group. *P < 0.05 vs. saline.

other agonists results in large increases in PGC-1α, (8, 14, 27, 28, 31, 40). It was particularly surprising that the β-adrenergic agonist used in these studies was the anabolic agent clenbuterol (8, 14, 27, 28, 40), which has the well-documented effect of decreasing the mitochondrial content of muscle, causing a shift in muscle fiber type to glycolytic/white/fast twitch, and markedly decreasing endurance exercise capacity (24, 29, 32, 38, 39, 48). Contrary to the reports by Ezaki’s group (27, 28, 40) Chinsomboon et al. (8), and Gerhart-Hines et al. (14), our results show that injection of clenbuterol, or of norepinephrine, does not result in increases in PGC-1α mRNA or protein or in mitochondrial enzymes. We were also unable to confirm the claim by Ezaki’s group that β-adrenergic blockade prevents the increase in PGC-1α induced by AICAR injection.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


Fig. 6. Anisomycin activates p38 MAPK in skeletal muscle, resulting in ATF2 phosphorylation and an increase in PGC-1α promoter activity. Treatment of C2C12 myotubes with 0.1 μM anisomycin for 30 min resulted in phosphorylation of p38 MAPK (A) and increased phosphorylation of ATF2 (B). C: activation of p38 MAPK with anisomycin does not result in phosphorylation of CREB. D: anisomycin treatment resulted in activation of the PGC-1α promoter in C2C12 myotubes transfected with a PGC-1α-luciferase construct. A dominant negative ATF2 blocked anisomycin-induced activation of the PGC-1α promoter; n = 8 per group. *P < 0.01 vs. saline; #P < 0.01 vs. wild type.


