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journal or publication title	Biochemical and biophysical research communications
volume	368
number	2
page range	323-328
year	2008-04
権利	(C)2008 Elsevier Inc.
URL	http://hdl.handle.net/2241/98745

doi: 10.1016/j.bbrc.2008.01.095

Acute exercise induces biphasic increase in respiratory mRNA in skeletal muscle

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Footnote: Mitochondria and p38 MAPK in skeletal muscle

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ABSTRACT

Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) promotes the expression of oxidative enzymes in skeletal muscle. We hypothesized that activation of the p38 MAPK (mitogen-activated protein kinase) in response to exercise was associated with exercise-induced PGC-1α and respiratory enzymes expression and aimed to demonstrate this under the physiological level. We subjected mice to a single bout of treadmill running and found that the exercise induced a biphasic increase in the expression of respiratory enzymes mRNA. The second phase of the increase was accompanied by an increase in PGC-1α protein, but the other was not. Administration of SB203580 (SB), an inhibitor of p38 MAPK, suppressed the increase in PGC-1α expression and respiratory enzymes mRNA in both phases. These data suggest that p38 MAPK is associated with the exercise-induced expression of PGC-1α and biphasic increase in respiratory enzyme mRNAs in mouse skeletal muscle under physiological conditions.

Key words: mitochondria, PGC-1α, p38 MAPK, single bout of exercise

INTRODUCTION

Endurance exercise increases the expression of oxidative enzymes in the mitochondria of skeletal muscle [1]. However, the precise mechanism by which this increase occurs is obscure. PGC-1α, a transcriptional coactivator cloned from a differentiated brown fat cell line [2], has recently been shown to play an important role in adaptive thermogenesis, glucose metabolism, mitochondrial biogenesis and muscle fiber type specialization [3]. Several lines of evidence are consistent with the notion that PGC-1α has a pivotal role in promoting oxidative capacity in skeletal muscle. Transient expression of the PGC-1α gene induces an increase in mitochondrial gene expression in several cell lines [2, 4-6]. Muscle-specific overexpression of PGC-1α in transgenic mice results in enhanced mitochondrial biogenesis and slow-twitch (type I) fiber formation[7]. Although endurance exercise induces PGC-1α mRNA and protein expression in rodents and humans [8-11], it is currently unknown whether exercise-induced PGC-1α expression in skeletal muscle is essential for mitochondrial protein expression in physiological conditions because the notion that PGC-1α induces mitochondrial protein expression was based on the findings of gain-of-function studies.

The p38 mitogen activated protein kinase (MAPK) pathway in skeletal muscle has been a focus of recent study in myogenic cell differentiation, glucose metabolism and energy expenditure [12-14]. Although exercise induces activation of p38 MAPK in rodents and humans, the downstream effects of the activation in skeletal muscle are currently unknown. Based on several previous findings, we hypothesized that activation of p38 MAPK in response to exercise might mediate PGC-1α expression, followed by expression of mitochondrial protein. For example, phosphorylated p38 MAPK can directly stimulate transcriptional factors of the PGC-1α gene, such as ATF2 and MEF2 [15-17]. Overexpression of MKK3E, an upstream kinase of p38 MAPK, or wild-type p38 isoforms stimulates PGC-1α promoter activity in C2C12 myocytes, whereas muscle-specific expression of MKK6E enhances PGC-1α protein expression in fast-twitch muscles [18]. These data suggest that p38 MAPK is associated with exercise-induced PGC-1α expression. However, it is uncertain whether activation of p38 MAPK stimulates PGC-1α gene transcription, followed by mitochondrial enzyme expression under physiological conditions. This is because 1) previous studies merely showed that exercise induced phosphorylation of p38 MAPK, or 2) the results were based on methodology involving overexpression.

In this study, we subjected mice to a single bout of treadmill running and investigated the effect of the exercise on expression of PGC-1α and respiratory enzymes. Then, to determine the effect of p38 MAPK activation on the expression of PGC-1α and respiratory enzymes under the physiological level, we used a pharmacological inhibitor of p38 MAPK, SB203580 (SB). We hypothesized that a single bout of exercise would induce expression of PGC-1α and respiratory enzymes through activation of p38 MAPK in skeletal muscle, as shown in overexpression studies.

MATERIALS AND METHODS

Animals

Female ICR mice (CLEA, Tokyo, Japan) aged 10 weeks were used. Food (Oriental Yeast, Tokyo, Japan) and water were provided *ad libitum*. The room temperature was maintained at 22 ± 1°C with a 12-h light (07:00 to 19:00) and 12-h dark (19:00 to 07:00) cycle. All protocols were approved by the Animal Experimental Committee of the University of Tsukuba.

Exercise Protocols

Mice were randomly assigned to either a non-exercised control group (n = 6) or exercise groups (n = 36). Six days before the experiment, all of the mice were acclimated for three days to a motor-driven treadmill by walking at a speed of 5-10 m/min for 15 min per day. Thirty-six mice then ran on the treadmill at 20 m/min for 90 min, and were anesthetized with an intraperitoneal injection of nembutal (0.1 mg/g body weight) immediately, or at 1, 3, 6, 12, or 24 h after exercise. Until anesthetization, food and water were provided *ad libitum*. Because the plantaris muscle is well recruited in treadmill running and has been used in several studies [8, 11, 18, 19], the muscle was dissected out quickly from each mouse, taking special care not to stretch it, frozen in

liquid nitrogen, and stored at -80°C until assay.

SB203580 administration

Forty-eight female ICR mice were divided into eight groups: control + saline, control + SB, immediately after running + saline, immediately after running + SB, 1 h after running + saline, 1 h after running + SB, 12 h after running + saline and 12 h after running + SB. Four groups of mice were administered SB dissolved in saline (25 mg/kg body weight) twice by intraperitoneal injection, 16 h and 1 h before treadmill running, whereas the others were administered the same volume of saline. The dose of SB employed in a previously reported study was used [20], and we verified its effect before conducting the present study. After treatment with SB or saline, the mice were run on the treadmill at 20 m/min for 90 min, and then immediately after, 1 h or 12 h after running, they were killed, and their plantaris muscles were harvested, frozen in liquid nitrogen, and stored at -80°C until assay. As all mice accomplished the exercise protocol, SB administration did not appear to affect running performance.

RNA isolation

Total RNA was extracted using the SV Total RNA Isolation System (Promega, WI,

USA) according to the manufacturer's protocol. Total RNA concentration was determined spectrophotometrically at 260 nm.

Reverse transcription and real-time PCR

First-strand cDNA was generated from 1μg of total RNA using ReverTra Ace α (TOYOBO, Osaka, Japan). Real-time PCR was performed using a 7500 Real Time PCR System (Applied Biosystems, CA, USA) and Power SYBR Green PCR Master Mix (Applied Biosystems). The PCR primers used were as: cyclophilin forward primer, 5'-CGATGACGAGCCCTTGG-3'; reverse, 5'-TCTGCTGTCTTTGGAACTTTGTC-3'; cytochrome *c* (CYTC) forward primer, 5'- GCTTATCGGCCACCCAAGTG-3'; reverse, 5'-GGGCTGCTGAGAGCTTGTTC-3'; cytochrome *c* oxidase subunit IV (COXIV) forward primer, 5'-TACTTCGGTGTGCCTTCGA-3'; reverse, 5'-TGACATGGGCCACATCAG-3'; ATP synthase β subunit (ATPsynβ) forward primer, 5'- CAGGCTATCTATGTGCCTGCTGAT-3'; reverse, 5'-GCATCCAAATGGGCAAAGG-3', carnitine palmitoyltransferase I (CPT-I) forward primer, 5'-ACCACATCCGCCAAGCA-3'; reverse, 5'-TTCCTCAGCTGTCTGTCTTGGA-3'. The PCR conditions for all genes consisted of one denaturing cycle at 95°C for 10 min, 40 cycles consisting of denaturing at 95°C for

15 s, and annealing and elongation at 60°C for 1 min. At the end of the PCR, the samples were subjected to dissociation curve analyses. The relative amounts of mRNA were determined by the standard curve method. Signals were normalized against cyclophilin mRNA. We also used S26 ribosomal protein as an internal control and this provided results similar to those when the PCR signals were normalized to cyclophilin. Cyclophilin and S26 mRNAs were not affected by either exercise or SB203580 (data not shown). All samples for each gene were run in duplicate.

Western blotting

For PGC-1α protein analysis, dissected plantaris muscles were homogenized and analyzed as described previously [19].

Protein kinase assay for p38 MAPK

p38 MAPK kinase activity was determined as described previously with some modifications [16]. In brief, *in vitro* kinase assays were performed at 37 °C for 30 min by using 10 μl of whole-muscle lysates, 2 μg of substrate ATF-2 Fusion Protein (purchased from Cell Signaling) and 250 μM ATP in 40 μl kinase reaction buffer. The ATF-2 fragment used in these experiments was a specific substrate for p38 MAPK [21].

An equivalent amount of 2× Laemmli sample buffer was added to terminate the reactions. Proteins were resolved with 10 % polyacrylamide gels, transfered to PVDF membrane, and detected by anti phospho-ATF-2 antibodies (Cell Signaling). The level of p38 MAPK in the cell lysates was detected by Western blotting with antibodies against p38 MAPK.

Statistical analysis

All data are expressed as means ± SE. The effect of treadmill running was analyzed using one-way ANOVA. Two-way ANOVA was used for SB203580 administration studies. If ANOVA indicated a significant difference, Tukey HSD was performed to determine the significance of the difference between means.

RESULTS AND DISCUSSION

Effect of a single bout of exercise on PGC-1α protein and respiratory enzyme mRNAs expression in mitochondria of mouse skeletal muscle

A 90-min bout of treadmill running induced transient PGC-1α protein expression 6 to 12 h after exercise, with an about 1.5-fold increase ($p < 0.05$ compared to pre group, Fig. 1A). This increase in PGC-1α expression returned to control levels within 24 h after exercise. The kinetics of the increase in PGC-1α protein is comparable to those in previous studies of human and rat muscle [9, 10].

Recent studies have indicated that PGC-1α has a pivotal role in mitochondrial biogenesis and that PGC-1α expression is a rate-limiting step for mitochondrial gene expression in skeletal muscle [10, 18, 22-24]. Therefore, we investigated temporal changes in the expression of respiratory enzyme (CYTC, COXIV and ATPsynβ) mRNAs in mouse skeletal muscle after a single 90-min bout of treadmill running. The exercise induced a biphasic increase in the expression of respiratory enzyme mRNAs in the plantaris muscle (Fig. 1B). The second phase increase (6 ~ 12 h after exercise) in respiratory enzyme mRNA expression was concomitant with an increase in PGC-1α protein expression level, but the first phase increase (1 h after exercise) showed slightly

but no such a clear correlation. These data suggested that skeletal muscle have another way to induce the expression of respiratory enzymes rather than PGC 1α expression in 1st phase.

It is surprising that a single bout of exercise induced a biphasic increase in the expression of respiratory enzyme mRNAs. Hildebrandt et al. and Pilegaard et al. reported that exercise stimulated the transcription of several genes expressed in mitochondria including pyruvate dehydrogenase kinase-4 (PDK4), uncoupling protein-3 (UCP3) and CPT-I in rat and human, respectively [9, 25]. However, these increases were not biphasic. To assess whether the biphasic increase after single bout of exercise is specific for respiratory enzyme mRNAs, we measured the mRNA expression of CPT-I after single bout of exercise. As shown in Fig 1B, CPT-I mRNA was increased 6 and 12 h after single bout of exercise (1.8-fold increase after 6 h and 1.7-fold increase after 12 h). The kinetics of CPT-I mRNA increase is transient as shown in previous studies [9, 25]. These results suggested that biphasic increase is specific to respiratory enzymes. The difference in post-exercise mitochondrial enzyme expression between the respiratory and CPT-I mRNAs might be explained for by differences in the transcriptional regulator involved. Several previous studies have suggested that transcription of respiratory enzymes is regulated by transcription factors such as,

Nuclear Respiratory Factor-1 and -2 (NRF-1 and 2) or Estrogen-related Receptor α [26, 27]. On the other hand, it has been considered that expression of PDK4, UCP3 and CPT-I is modulated by members of the peroxisome proliferators-activated receptor family [28-30].

Effect of inhibition of p38 MAPK activation in response to a single bout of exercise on expression of PGC-1α and respiratory enzymes

Many previous reports have demonstrated that exercise or contraction activates MAPK signaling [21, 31-35], and that MAPK activity increases immediately after an acute bout of exercise [21, 34] or electrical stimulation [35]. We speculated that MAPK, especially p38, mediates increases in PGC-1α expression and respiratory mRNA after acute treadmill running. Therefore, we assessed temporal changes in p38 MAPK activity after acute treadmill running by kinase assay using ATF-2 Fusion Protein as a specific substrate of p38 MAPK in mouse skeletal muscles. Activity of p38 MAPK was increased 0 h after treadmill running and returned to the base level after 12 h (Fig. 2A).

To determine whether p38 MAPK activation is required for induction of the PGC-1α and respiratory enzyme gene after treadmill running, mice were pretreated with SB, a pharmacological inhibitor of p38 MAPK, and then expression levels of

PGC-1α and respiratory enzyme mRNAs in the plantaris muscle were determined after treadmill exercise. As shown in Fig. 2A, p38 MAPK activity in the plantaris muscle was increased 0 h after cessation of treadmill running and this increase was inhibited about 50 % by pretreatment with SB. This is the first report that can inhibit exercise-induced activation of p38 MAPK in skeletal muscle *in vivo*. Administration of SB also suppressed the exercise-related induction of PGC-1α protein expression completely in the plantaris muscle (12 h after exercise, Fig 2B). This result suggested that exercise-induced increase in PGC-1α expression required for p38 MAPK activation. As stated above, acute exercise induced a biphasic increase in the expression of respiratory enzyme mRNAs. The first phase was not concomitant with PGC-1α protein expression, whereas the second phase (6 and 12 h after exercise) was accompanied by an increase in PGC-1α protein. Administration of SB also suppressed both phase increase in respiratory mRNA (Fig 3). These finding suggest that p38 MAPK is a central mediator of exercise-induced respiratory enzyme expression in skeletal muscle. However, how does p38 MAPK mediate the expression of respiratory enzyme mRNAs in both phases of the increase? In the second phase, SB was able to inhibit the exercise-induced increase in PGC-1α protein. Therefore, it can be thought that p38 MAPK mediates the expression of respiratory enzyme mRNAs in the second phase through induction of

PGC-1α expression. On the other hand, it is more difficult to account for the first phase of the increase. One possibility is phosphorylation of PGC-1α by p38 MAPK. p38 MAPK phosphorylates PGC-1α at three residues (T262, S265 and T298), and this increases the stability and half-life of the molecule [12]. Another study has demonstrated that p160 myb binding protein (p160MBP) binds this region (amino acids 200-403) and represses the activity of PGC-1α as a transcriptional coactivator. When phosphorylated in this region, p160MBP dissociates from PGC-1α, and PGC-1α becomes able to bind transcriptional factors such as NRF-1 and initiate transcriptional activity [36, 37]. NRF-1 is an important transcription factor regulating the expression of mitochondrial enzymes [38, 39]. Currently, it is unknown whether exercise induces phosphorylation of PGC-1α via p38 MAPK pathways, and this issue will require future study.

ACKNOWLEDGMENT

This work was supported in part by Grants #15200048 and #17650189 and in part by the Nishihira/Tsukuba Project of the 21st Century of COE (Center of Excellence) program from Ministry of Education, Culture, Sports, Science and Technology.

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FIGURE LEGENDS

Figure 1

Effect of a single bout of exercise on PGC-1α protein expression (A) and mitochondrial mRNA expression (B). The pre group was assigned a value of 1.0; all other values are expressed relative to this value. All values are reported as means ± SE (n = 6). * significantly different from the pre group ($p < 0.05$).

Figure 2

Administration of 25 mg/kg SB203580 inhibits exercise-induced activation of p38 MAPK and PGC-1α expression in mouse skeletal muscle. (A) p38 MAPK kinase assay and (B) Western blotting using anti-PGC1 antibody. All values are reported as means ± SE (n = 6). * significantly different from the control + saline group, # significantly different from the 12 h + saline group ($p < 0.01$).

Figure 3

Expression of mRNAs for CYTC, COXIV and ATPsynβ in mouse plantaris muscle. All values are reported as means ± SE (n = 6). * significantly different from the control + saline group, # significantly different from the 1 h + saline group, $ significantly different from the 12 h + saline group ($p < 0.05$).

Fig. 1

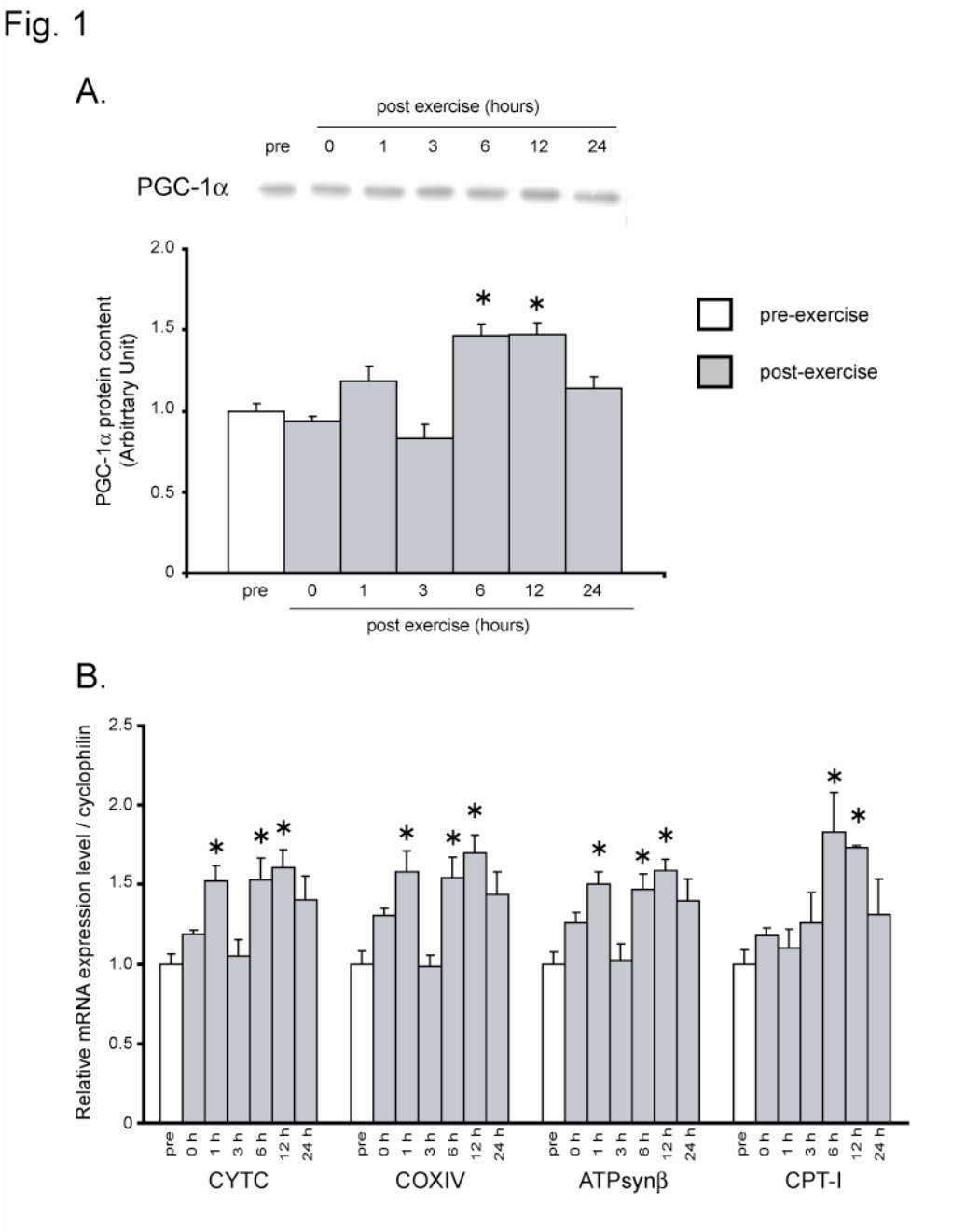
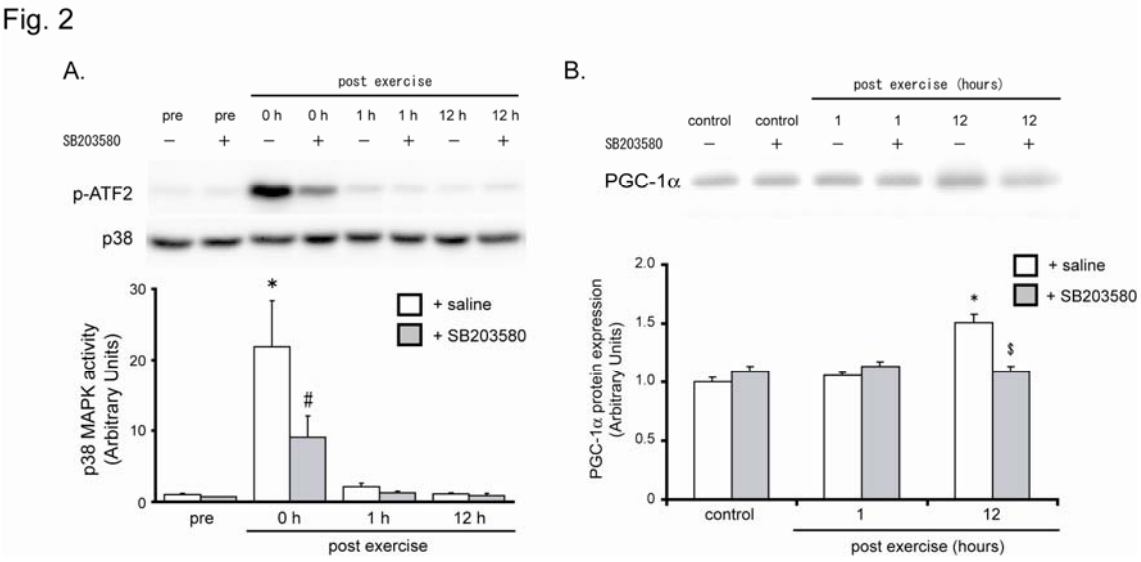

A.
post exercise (hours)
pre 0 1 3 6 12 24
PGC-1α
PGC-1α protein content (Arbitrtary Unit)
2.0
1.5
1.0
0.5
0
pre-exercise
post-exercise
pre 0 1 3 6 12 24
post exercise (hours)
B.
Relative mRNA expression level / cyclophilin
2.5
2.0
1.5
1.0
0.5
0
pre 0 h 1 h 3 h 6 h 12 h 24 h
CYTC
COXIV
ATPsynβ
CPT-I

Fig. 2


A.
post exercise
pre
pre
0 h
0 h
1 h
1 h
12 h
12 h
SB203580
−
+
−
+
−
+
−
+
p-ATF2
p38
p38 MAPK activity (Arbitrary Units)
30
20
10
0
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+ saline
+ SB203580
pre
0 h
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12 h
post exercise
B.
post exercise (hours)
control
control
1
1
12
12
SB203580
−
+
−
+
−
+
PGC-1α
PGC-1α protein expression (Arbitrary Units)
2.0
1.5
1.0
0.5
0
*
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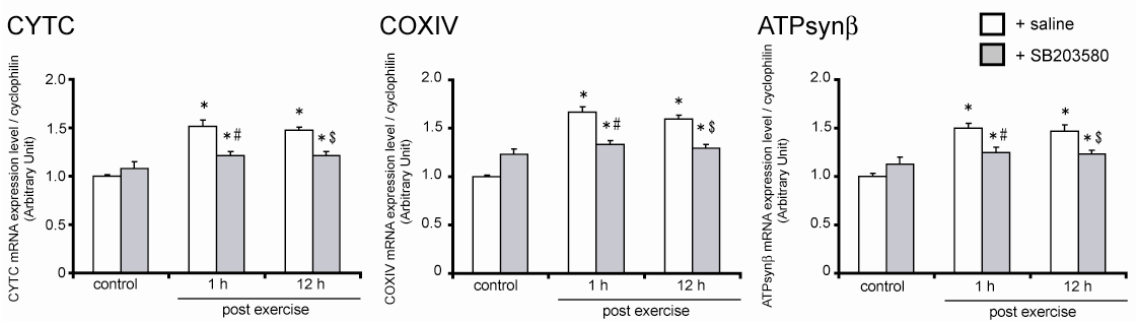
+ saline
+ SB203580
control
1
12
post exercise (hours)

Fig. 3


CYTC
COXIV
ATPsynβ
+ saline
+ SB203580
CYTC mRNA expression level / cyclophilin (Arbitrary Unit)
COXIV mRNA expression level / cyclophilin (Arbitrary Unit)
ATPsynβ mRNA expression level / cyclophilin (Arbitrary Unit)
2.0
1.5
1.0
0.5
0
control
1 h
12 h
post exercise
*
*#
*$