Effects of acute bouts of running and swimming exercise on PGC-1α protein expression in rat epitrochlearis and soleus muscle

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Terada, Shin, and Izumi Tabata. Effects of acute bouts of running and swimming exercise on PGC-1α protein expression in rat epitrochlearis and soleus muscle. Am J Physiol Endocrinol Metab 286: E208–E216, 2004. —The purpose of this study was to elucidate the mechanisms underlying low-intensity exercise-induced peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) protein expression in rat skeletal muscles. Rats (5–6 wk old) swam without a load and ran on the treadmill at a speed of 13 m/min, respectively, in two 3-h sessions separated by 45 min of rest. PGC-1α content in epitrochlearis muscle (EPI) was increased by 75 and 95%, immediately and 6 h after swimming, respectively, with no increase in PGC-1α content in the soleus (SOL). After running, PGC-1α content in EPI was unchanged, whereas a 107% increase in PGC-1α content was observed in SOL 6 h after running. Furthermore, in EPI and SOL as well as other muscles (triceps, plantaris, red and white gastrocnemius), PGC-1α expression was enhanced concomitantly with reduced glycogen postexercise, suggesting that expression of PGC-1α occurs in skeletal muscle recruited during exercise. PGC-1α content in EPI was increased after 18-h in vitro incubation with 0.5 mM 5-aminimidazole-4-carboxamide ribonucleoside (AICAR) and 4 mM caffeine. However, AICAR incubation did not affect PGC-1α content in the SOL, whereas caffeine incubation increased it. These results suggest that exercise-induced PGC-1α expression in skeletal muscle can be mediated by at least two exercise-induced signaling factors: AMPK activation and Ca²⁺ elevation. The number of factors involved (both AMPK and Ca²⁺, or Ca²⁺ only) in exercise-induced PGC-1α expression may differ among muscles.

Peroxisome proliferator-activated receptor-γ coactivator-1α; adenosine 5′-monophosphate-activated protein kinase; calcium; 5-aminimidazole-4-carboxamide ribonucleoside; caffeine; low-intensity exercise

PHYSICAL TRAINING INCREASES THE ACTIVITY OF oxidative enzymes in the mitochondria in skeletal muscle (13). However, the precise mechanisms by which this increase in activity occurs are not known (16, 17). Lehman et al. (22) demonstrated that peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) promotes cardiac mitochondrial biogenesis. Furthermore, it has been suggested that this transcriptional coactivator is involved in mitochondrial biogenesis in skeletal muscle (16, 17, 44). Recently, we (38) and Baar et al. (4) reported that an acute bout of 6-h low-intensity prolonged swimming exercise significantly increased PGC-1α mRNA and protein expression in rat forelimb muscles, respectively. In addition, we (38) also showed that electrical stimulation of the rat epitrochlearis muscle in vitro increases PGC-1α mRNA expression, suggesting that the stimuli evoked in the recruited muscle directly enhance PGC-1α expression during exercise. However, there are several factors that are known to emerge during exercise, and these may possibly induce skeletal muscle PGC-1α expression. These factors include β-adrenergic receptor stimulation, which has been reported to enhance PGC-1α expression in response to cold exposure (33, 44). Therefore, for the purpose of evaluating the contribution of contraction and other nonspecific factors to exercise-induced PGC-1α expression in skeletal muscle, in the present investigation we observed changes in PGC-1α protein content after different modes of exercise (running vs. swimming), in which different muscles are recruited and in which the humoral effects might be virtually similar.

Previously, we found that 18-h incubation with 0.5 mM 5-aminimidazole-4-carboxamide ribonucleoside [AICAR, a 5′-AMP-activated protein kinase (AMPK) activator] induced approximately twofold higher PGC-1α mRNA expression in rat epitrochlearis muscle compared with the control muscle (38). In addition, Ojuka et al. (28) recently suggested that elevated intracellular Ca²⁺ concentration ([Ca²⁺]i) (39) induces skeletal muscle mitochondrial biogenesis, which might be regulated by PGC-1α. Therefore, for the purpose of elucidating the mechanism related to the swimming and running exercise-induced PGC-1α protein expression, we observed the effects of AMPK activation and of the increase in [Ca²⁺], on PGC-1α expression in the rat epitrochlearis and soleus muscles.

MATERIALS AND METHODS

Materials

Antibodies to PGC-1α and Sp1 protein were purchased from Calbiochem (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All other biochemicals, not mentioned here, were purchased from Sigma (St. Louis, MO).

Exercise Protocols

Animal care. All rats used for the present investigation were purchased from Crea (Tokyo, Japan). The animals were housed in rooms with light from 7 AM to 7 PM and were maintained on an ad libitum diet of standard chow and water. Room temperature was maintained at 20–22°C.

Low-intensity prolonged swimming and running exercise. Five- to six-week-old male Sprague-Dawley rats with body weights of 150–
170 g were used in this experiment. During low-intensity swimming exercise, the rats swim without a load for 6 h in two 3-h sessions separated by 45 min of rest (4, 34, 40). Eight rats swim simultaneously in a barrel filled to a depth of 50 cm and with an average surface area of 190 cm²/rat. The water temperature was maintained at 35°C during the swimming exercise. A period of 5 days of training involving this swimming exercise was shown to induce substantial increases in δ-aminolevulinate synthase, citrate synthase, cytochrome oxidase subunit 1, cytochrome c, and GLUT4 protein concentrations, suggesting that this exercise is a potent stimulus for mitochondrial biogenesis (4). Thus we adopted this swimming exercise program for the present investigation.

From the physiological point of view, the overall effect of a specific exercise generally depends on the exercise intensity and exercise time. Furthermore, the maximal exercise time is dependent on exercise intensity. In this context, for the specific purpose of comparing the effects of running exercise with those of swimming exercise on PGC-1α expression in muscle, an exercise intensity that exhausts the rats over the same duration as the swimming exercise should be used in the running exercise protocol. Therefore, 5- to 6-wk-old male Sprague-Dawley rats with body weights of 150–170 g ran on a rodent treadmill (Natsume, Tokyo, Japan) at the intensity (speed: 13 m/min, grade: 0%) that was experimentally found to exhaust the rat ~6 h after the start of the running exercise. Actually, the rats ran for 6 h in two 3-h sessions separated by 45 min of rest, the same protocol as that of the swimming exercise described above. Immediately after, 6 h after, or 18 h after the swimming and running exercises ended, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and the epitrochlearis and soleus muscles were dissected out. Age-matched sedentary control rats were kept in cages until they were killed. For muscle preparation, the epitrochlearis and soleus muscles were chosen because they have the following characteristics. 1) Epitrochlearis and soleus muscles have been shown to be recruited during swimming and running exercise, respectively. This is evidenced by glycogen depletion, stimulation of glucose transport (3, 20), and increased electromyographic activity (37) in response to a bout of exercise and adaptive increases in GLUT4 and hexokinase (19, 34, 40). 2) The muscles can be utilized in an in vitro incubation experiment, which is a valuable tool for elucidating the potential mechanisms by use of pharmacological approaches (18, 26, 45).

In subsequent experiments, to further determine the relationship between the muscle recruitments during exercise and PGC-1α protein expression, the effects of low-intensity prolonged swimming and running exercise on muscle glycogen concentrations and PGC-1α protein expression in various rat skeletal muscles were observed. Five- to six-week-old male Sprague-Dawley rats with body weights of 150–170 g were used in this experiment. The rats performed the low-intensity prolonged swimming or running exercise we have described. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and the epitrochlearis, triceps, soleus, plantaris, and red and white regions of the gastrocnemius muscles were dissected out immediately after and 6 h after the swimming and running exercises, respectively, for measurements of muscle glycogen concentration and PGC-1α protein expression. Age-matched sedentary control rats were kept in cages until they were killed.

Effect of Low-Intensity Prolonged Swimming and Running Exercise on PGC-1α Protein Content in Skeletal Muscle of Younger Rats

In the present investigation, relatively younger rats with body weights of 50–60 g were used in vitro experiments, compared with rats used in the in vivo swimming and running exercise experiments. Because the weight of single muscles of the young rats that were usually used for our exercise experiments were not high enough for measuring PGC-1α protein with a nucleus extraction procedure, we used older and heavier rats for the purpose of minimizing the number of rats killed. However, in the additional experiment, we also observed the effects of the exercise on rat skeletal muscle in younger rats. In that experiment, three to four muscles were homogenized together.

Male Sprague-Dawley rats with body weights of 100–110 g were used in this experiment. During low-intensity swimming exercise, the rats swim without a load for 6 h in two 3-h sessions separated by 45 min of rest (4, 34, 40). Nine rats swim simultaneously in a barrel filled to a depth of 50 cm and with an average surface area of 170 cm²/rat. The water temperature was maintained at 35°C during the swimming exercise. During low-intensity prolonged running exercise, the rats ran on a rodent treadmill (Natsume, Tokyo, Japan) at a speed of 11 m/min (0% grade) for the same duration as in the low-intensity swimming exercise (6 h in two 3-h sessions separated by 45 min of rest). Six hours after the swimming and running exercises ended, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and the epitrochlearis and soleus muscles were dissected out. Age-matched sedentary control rats were kept in cages until they were killed.

In Vitro Epitrochlearis Muscle Incubation

For the purpose of elucidating the mechanism related to the swimming and running exercise-induced PGC-1α protein expression, we adopted in vitro pharmacological approaches that used rat epitrochlearis and soleus muscles. The epitrochlearis muscles were dissected out from 4-wk-old male Sprague-Dawley rats with body weights of 100–110 g. The epitrochlearis is a small, thin muscle, only 20 fibers thick, weighing 10-15 mg in rats of this size. This short diffusion distance makes it possible to maintain adequate ATP and creatine phosphate levels during prolonged incubation in vitro (26). Previous studies demonstrated the suitability of the epitrochlearis muscle for in vitro incubation experiments (26, 29, 30, 45). After dissection, the muscles were incubated for 18 h in 25-mL stoppered Erlenmeyer flasks containing 3 ml of oxygenated tissue culture medium, as described previously (29, 30, 38). The culture medium consisted of MEM-α (GIBCO BRL 12000–063), 10% fetal bovine serum (GIBCO BRL), 50 μU/ml purified human insulin (Eli Lilly), 100 μU/ml penicillin (GIBCO BRL), 100 μg/ml streptomycin (GIBCO BRL), and 0.25 μg/ml fungizone (Calbiochem). The incubation medium was sterilized by filtration through 0.2-μm Millipore filters. The flasks were gassed continuously with 95% O₂-5% CO₂ throughout the incubation. The medium was replaced with fresh medium every 6 h. For the purpose of observing the effects of AMPK activation on PGC-1α protein expression, muscles were randomly incubated with or without AMPK activator (0.5 mM AICAR).

Second, for the purpose of clarifying the effect of Ca²⁺ on PGC-1α expression, muscles were incubated in the presence or absence of 4 mM caffeine. Caffeine is shown to release Ca²⁺ from the sarcoplasmic reticulum (SR) (31). After incubation, the muscles were washed in 3 ml of PBS for 10 min, blotted, clamp-frozen, and stored at −80°C until they were used for the measurement of PGC-1α protein content and muscle glycogen concentration.

In Vitro Soleus Muscle Incubation

Three-week-old male Sprague-Dawley rats with body weights of 50–60 g were used in this experiment. Without use of a splitting procedure, intact soleus muscles dissected from rats with body weights of 100–120 g were shown to be unsuitable for an in vitro incubation experiment because of their thickness, which prevents the perfusion of glucose and oxygen into the inside of the muscle (12). Therefore, we used intact soleus muscle dissected from rats whose body weight and soleus muscle weight were 50–60 g and ~15–20 g, respectively. This size of the soleus has been verified to be metabolically viable and was used in an in vitro glucose transport experiment (18). The soleus muscles were dissected out and incubated for 18 h in glass vials in a shaking incubator maintained at 35°C. All muscles
were mounted on holders with two clips to keep them at resting length. Muscles were incubated in the tissue culture medium in the presence of either 0.5 mM or 5 mM AICAR, 4 mM caffeine, or vehicle (control). The flasks, containing 8 ml of medium, were gassed continuously with 95% O2-5% CO2 throughout the incubation. The incubation medium and protocols were identical with those used in the incubation of the epitrochlearis muscle. After incubation, the muscles were blotted, clamp-frozen, and stored at −80°C until they were used for the measurement of PGC-1α protein content and muscle glycogen concentration.

Measurement of PGC-1α Protein Content

Because PGC-1α is localized in the cell nucleus (33), nuclear extracts were prepared by the method of Blough et al. (8), as modified by Baar et al. (4). Approximately 70 mg of skeletal muscle tissue were pulverized into small pieces and then incubated on ice for 15 min with buffer 1, containing 100 mM potassium chloride (KCl), 5 mM EGTA, 5 mM sodium pyrophosphate (NaP2O7), 5 mM magnesium chloride (MgCl2), 2.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.25 μg/ml aprotinin, 10 μg/ml leupeptin, 0.4 M sodium orthovanadate (NaVO4), and 40 mM okadaic acid. After a brief 30-s centrifugation (Chivitan, Millipore), the pellets were incubated on ice for 15 min with buffer 2, containing 50 mM KCl, 5 mM EGTA, 1 mM NaP2O7, 5 mM MgCl2, 250 mM sucrose, 2.5 mM DTT, 1 mM PMSF, 12.5 μg/ml aprotinin, 10 μg/ml leupeptin, 0.4 mM NaVO4, and 40 mM okadaic acid. After a second brief 30-s centrifugation (Chivitan, Millipore), the pellets were homogenized in 1 ml of homogenization buffer containing 10 mM Tris, 1 mM EDTA, 5 mM MgCl2, 1.4 M sucrose, 2.5 mM DTT, 1 mM PMSF, 12.5 μg/ml aprotinin, 10 μg/ml leupeptin, 0.4 mM NaVO4, and 40 mM okadaic acid, pH 7.4. The homogenates were centrifuged for 10 min at 2,000 rpm. The pellets were resuspended in 1 ml of homogenization buffer and then were filtered through one layer of cheesecloth. After 5-min centrifugation at 10,000 g, the pellets were resuspended and incubated for 1 h with buffer 3, containing 31.5% (wt/vol) glycerol, 0.2 mM EDTA, 1.5 mM MgCl2, 10 mM HEPES, 0.45 M sodium chloride, 0.5% (wt/vol) Triton X-100, 75 mM KCl, 2.5 mM DTT, 1 mM PMSF, 12.5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM NaVO4, and 0.2 μM okadaic acid, pH 7.9. After centrifugation for 15 min at 10,000 g, the supernatant, designated as the nuclear extract, was frozen and stored at −80°C.

The nuclear extracts were incubated for 30 min at 37°C in Laemmli sampling buffer. SDS-PAGE was performed according to Laemmli (21) with a 0.56 M (4% wt/vol) stacking gel and a 0.98 M (7% wt/vol) resolving gel. Immunoblotting of electrophoresis gels was performed according to H9251 Blotting as described previously (40). Proteins in the gels were electrophoretically transferred to polyvinylidene difluoride sheets in a transfer buffer. The sheets were incubated successively with rabbit polyclonal antibodies to PGC-1α for 20 h and with 125I-labeled protein A for 24 h at 4°C. The PGC-1α antibody recognizes the COOH-terminal (777–797) amino acid of the PGC-1α protein. Autoradiography was performed with Fuji XAR film at −80°C for 4–12 h. To quantify the PGC-1α protein content, we cut out pieces of sheet containing the PGC-1α protein and counted the radioactivity using a γ-counter. To compare the amount of PGC-1α protein in different sheets, we applied a fixed amount of nuclear extract from rat skeletal muscles to each gel as a pooled control, and the radioactivity of the corresponding bands was normalized. In preliminary experiments, the precision of the assay was satisfactory; the coefficient of variation (CV) for PGC-1α protein measurement was <3%. To confirm that nuclear extracts could be recovered successfully, we also performed Western blotting with another nuclear transcription factor, Sp1 (Fig. 1A).

Measurement of Muscle Glycogen Concentration

Muscle glycogen concentrations were determined by enzymatic methods according to Lowry and Passonneau (23) after acid hydrolysis.

Statistics

In the experiments that examined the effects of acute bouts of swimming and running exercise on PGC-1α protein expression in rat epitrochlearis and soleus muscles at various time points, and in in vitro incubation experiments, data for PGC-1α protein content were expressed as a fold expression relative to the mean values observed in each control group. For the other experiments, in which the PGC-1α protein content in various skeletal muscles was observed after the exercises, we presented the data for PGC-1α protein content as a fold expression relative to the mean values observed in the epitrochlearis muscle from the control group of rats. All values were expressed as means ± SD. Statistical comparisons were made by a two-tailed t-test and one-way ANOVA (Jandel Sigma Stat). Statistical significance was defined as P < 0.05.

RESULTS

Effects of Low-Intensity Prolonged Swimming and Running Exercises on PGC-1α Protein Content in Rat Epitrochlearis and Soleus Muscle

PGC-1α protein content in exercised rat epitrochlearis muscle, compared with that of the control muscle, significantly increased by 75, 95, and 60% immediately after, 6 h after, and 18 h, respectively, after low-intensity prolonged swimming exercise (P < 0.001, Fig. 1A). No significant increase in PGC-1α protein content in rat soleus muscle was observed at
any time point after the low-intensity prolonged swimming exercise (Fig. 1B).

PGC-1α protein content in rat soleus muscle was significantly elevated by 107 and 87% at 6 and 18 h, respectively, after the low-intensity prolonged running exercise (P < 0.001; Fig. 2B). Low-intensity prolonged running exercise did not affect PGC-1α protein content in rat epitrochlearis muscle at any time point (Fig. 2A).

**PGC-1α Protein Content in Various Rat Skeletal Muscles During the Basal Condition**

In the nonexercised control group of rats, higher levels of PGC-1α protein were found in the red region of gastrocnemius and soleus muscles. These levels were 177 and 166%, respectively, of those found in epitrochlearis muscle (Fig. 3). The lowest level of PGC-1α protein content was found in the white region of gastrocnemius muscle (Fig. 3).

**Effects of Low-Intensity Prolonged Swimming and Running Exercise on PGC-1α Protein Content and Muscle Glycogen Concentration in Various Skeletal Muscles**

As shown in Fig. 3, low-intensity prolonged swimming exercise significantly increased PGC-1α protein content not only in epitrochlearis muscle but also in the triceps and the red region of gastrocnemius muscles by 96 and 70%, respectively (P < 0.001), at 6 h after the low-intensity swimming exercise. No significant increase in PGC-1α protein content was observed in soleus or plantaris muscles or in the white portion of gastrocnemius muscles at the same time point (Fig. 3).

At 6 h after the low-intensity prolonged running exercise, 69, 86, and 61% increases in PGC-1α protein content were observed in the soleus and plantaris muscles and in the red region of the gastrocnemius muscles, respectively (P < 0.001, Fig. 3). The low-intensity running exercise did not affect PGC-1α protein content in the epitrochlearis, triceps, or the white region of the gastrocnemius muscles (Fig. 3).

As shown in Table 1, low-intensity prolonged swimming exercise significantly reduced muscle glycogen concentrations in the rat epitrochlearis and triceps and in the red region of the gastrocnemius muscles (P < 0.001, P < 0.001, and P < 0.01, respectively). No significant decrease in muscle glycogen concentration was observed in the soleus or plantaris muscles or in the white region of the gastrocnemius muscles (Table 1).

Muscle glycogen concentration in the soleus and plantaris muscles and in the red region of the gastrocnemius muscles
significantly decreased after the low-intensity prolonged running exercise ($P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively, Table 1). There were no significant changes in muscle glycogen concentrations in the epitrochlearis, triceps, or white region of the gastrocnemius muscles (Table 1).

**Effects of Low-Intensity Prolonged Swimming and Running Exercise on PGC-1α Protein Content in Epitrochlearis and Soleus Muscles of Younger Rats**

The PGC-1α protein content in the epitrochlearis muscle of the younger rats, compared with that of the control muscle, significantly increased by 87% at 6 h after low-intensity prolonged swimming exercise ($P < 0.001$; Fig. 4). No significant increase in PGC-1α protein content was observed in rat soleus muscle at the same time point after the low-intensity prolonged swimming exercise (Fig. 4). In contrast, the PGC-1α protein content in the soleus muscle of the younger rats was significantly elevated by 50% at 6 h after the low-intensity prolonged running exercise ($P < 0.001$; Fig. 4). Low-intensity prolonged running exercise did not affect PGC-1α protein content in the epitrochlearis muscle of the younger rats at the same time point (Fig. 4).

**Effects of 18-h Incubation with AICAR and Caffeine on PGC-1α Protein Content in Rat Epitrochlearis Muscle**

As shown in Fig. 5, 18-h incubation with 0.5 mM AICAR induced PGC-1α protein content in rat epitrochlearis muscle that was 32% higher than that observed in the control muscle ($P < 0.01$). Exposure to 4 mM caffeine for 18 h also induced a 72% increase in PGC-1α protein content in rat epitrochlearis muscle ($P < 0.001$; Fig. 4).

**Effects of 18-h Incubation with AICAR and Caffeine on PGC-1α Protein Content in Rat Soleus Muscle**

Caffeine incubation induced a 37% higher PGC-1α protein content in rat soleus muscle than in the control muscles ($P < 0.01$). However, PGC-1α protein content in rat soleus muscles that were incubated with 0.5 mM AICAR did not differ from that observed in control muscles. Furthermore, exposure to a high concentration of AICAR (5 mM) for 18 h did not affect PGC-1α protein content in rat soleus muscle (control vs. 5 mM AICAR: 1.00 ± 0.15-fold vs. 0.97 ± 0.16-fold expression).

### Table 1. Effects of acute bouts of running and swimming exercise on glycogen concentrations in rat skeletal muscles

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Glycogen Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitrochlearis</td>
<td>27.4±4.3</td>
</tr>
<tr>
<td>Swimming</td>
<td>10.7±2.3†</td>
</tr>
<tr>
<td>Control</td>
<td>29.4±5.4</td>
</tr>
<tr>
<td>Running</td>
<td>25.2±5.6</td>
</tr>
<tr>
<td>Triceps</td>
<td>27.5±2.5</td>
</tr>
<tr>
<td>Swimming</td>
<td>15.7±2.8†</td>
</tr>
<tr>
<td>Control</td>
<td>29.2±3.6</td>
</tr>
<tr>
<td>Running</td>
<td>25.2±3.3</td>
</tr>
<tr>
<td>Soleus</td>
<td>14.4±3.9</td>
</tr>
<tr>
<td>Swimming</td>
<td>14.0±3.2</td>
</tr>
<tr>
<td>Control</td>
<td>17.7±0.9</td>
</tr>
<tr>
<td>Running</td>
<td>8.6±1.4†</td>
</tr>
<tr>
<td>Plantaris</td>
<td>24.8±4.4</td>
</tr>
<tr>
<td>Swimming</td>
<td>20.7±2.7</td>
</tr>
<tr>
<td>Control</td>
<td>28.9±3.2</td>
</tr>
<tr>
<td>Running</td>
<td>19.7±1.9†</td>
</tr>
<tr>
<td>Red Gastrocnemius</td>
<td>23.9±4.5</td>
</tr>
<tr>
<td>Swimming</td>
<td>15.8±2.6*</td>
</tr>
<tr>
<td>Control</td>
<td>25±3.7</td>
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<tr>
<td>Running</td>
<td>18.6±2.4*</td>
</tr>
<tr>
<td>White Gastrocnemius</td>
<td>32.6±2.2</td>
</tr>
<tr>
<td>Swimming</td>
<td>30.7±5.2</td>
</tr>
<tr>
<td>Control</td>
<td>35.3±3.9</td>
</tr>
<tr>
<td>Running</td>
<td>33.8±2.0</td>
</tr>
</tbody>
</table>

Values are means ± SD in μmol/g tissue. *$P < 0.01$, †$P < 0.001$: significantly different from values obtained in control muscles.

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Fig. 4. PGC-1α protein content in epitrochlearis and soleus muscle of younger rats at 6 h after low-intensity prolonged swimming and running exercise. Values are means ± SD. Significantly different ($P < 0.001$) from ***values obtained in control and running groups; §§§values obtained in control and swimming groups.

Fig. 5. Effects of 18-h incubation with 0.5 mM AICAR and 4 mM caffeine on PGC-1 protein content in rat epitrochlearis (A) and soleus (B) muscle. Values are means ± SD. **$P < 0.01$ and ***$P < 0.001$, significantly different from values obtained in control group.
Effects of 18-h Incubation with AICAR and Caffeine on Muscle Glycogen Concentration in Rat Epitrochlearis and Soleus Muscles

As shown in Table 2, incubation for 18 h in oxygenated culture medium with vehicle (control) did not affect muscle glycogen concentrations in either the epitrochlearis or soleus muscles. AICAR incubation induced a 31% increase in muscle glycogen concentrations in rat epitrochlearis muscle ($P < 0.05$) but not in soleus muscle. Muscle glycogen concentrations in the epitrochlearis and soleus muscles were significantly reduced by 28% ($P < 0.01$) and 24% ($P < 0.05$), respectively, after 18-h incubation with caffeine (Table 2).

**DISCUSSION**

The present investigation demonstrated that an acute bout of low-intensity exercise increased the PGC-1α protein content in rat skeletal muscle in a site-specific manner, confirming our previous conclusion that PGC-1α expression in skeletal muscle after exercise is induced mainly by muscle activity/contraction (38). Furthermore, the results obtained in the present investigation suggest that exercise-induced PGC-1α expression in rat skeletal muscle may be mediated by at least two exercise-induced biochemical signaling factors, including AMPK activation and the elevation of Ca$^{2+}$. In addition, the results may suggest that the number of factors (both AMPK and Ca$^{2+}$, or Ca$^{2+}$ only) involved in exercise-induced PGC-1α expression may differ among specific muscles.

Consistent with the increase in the mRNA (38), the present investigation demonstrated that the increased expression of PGC-1α protein content in rat epitrochlearis muscle occurred rather rapidly after the swimming exercise (Fig. 1), as was reported by Baar et al. (4). In contrast with this finding, Tunstall et al. (41) reported that PGC-1α mRNA was unchanged in the human vastus lateralis muscle both immediately and 3 h after exercise. The reasons for the disparate results between the two studies are not clear but might be related to differences in the subjects (human, rat), mode (cycling vs. swimming or running), duration (1 vs. 6 h), and/or intensity (63% of peak oxygen uptake for the human vs. ~50% maximum oxygen uptake for the rat) of exercise between the two studies. Another possibility is that, for the 24 h before each trial, the subjects of the study by Tunstall et al. consumed a high carbohydrate diet, which may have elevated the preexercise muscle glycogen levels that might affect the expression of exercise-responsive genes (32).

Electrical stimulation of epitrochlearis muscle in vitro induced PGC-1α expression (38), suggesting that stimuli evoked in the recruited muscle directly enhance PGC-1α expression during exercise. However, several factors are known to emerge during exercise and possibly to induce skeletal muscle PGC-1α expression, including the stimulation of β-adrenergic receptors (33, 44). In the present investigation, we found an exclusively site-specific increase in PGC-1α expression after specific exercises that recruited different muscle groups. The electromyographic activity in rat soleus muscle recorded during swimming was reported to be lower than that observed during treadmill running (37). In addition, Armstrong and Taylor (3) demonstrated that muscle glycogen concentration in the rat forelimb muscle did not significantly change during treadmill running. These previous studies suggest that, in the rat, swimming exercise recruits mainly forelimb muscles, probably including the epitrochlearis muscle, whereas running exercise recruits mainly hindlimb muscles, including the soleus muscle. In the present investigation we found, for the first time, that acute bouts of prolonged swimming and running exercise affect the PGC-1α expression differently in rat epitrochlearis and soleus muscles (Figs. 1 and 2). Furthermore, for the purpose of confirming the relationships between the metabolic activity of the muscle and the PGC-1α expression induced by the activity, in the subsequent experiment we observed the changes in muscle glycogen concentrations and PGC-1α protein content in various skeletal muscles after low-intensity prolonged swimming and running exercises. Consequently, at least among the muscles tested in the present investigation, substantial increases in PGC-1α protein were observed exclusively in rat skeletal muscles in which glycogen concentrations significantly decreased after the swimming and running exercises (Table 1 and Fig. 3). On the basis of these findings, it might be concluded that the contribution made by exercise-related factors other than muscle contraction is negligible in terms of the PGC-1α expression of skeletal muscle after single bouts of exercise.

We previously reported that 18-h incubation with AICAR increased PGC-1α mRNA expression in rat epitrochlearis muscle (38). As was expected, the present investigation showed that AICAR in vitro induced enhanced PGC-1α protein expression in the muscle (Fig. 3). This result further suggests that AMPK activation during exercise is one of the postulated stimuli that induce PGC-1α expression in the rat epitrochlearis muscle. In contrast to the epitrochlearis muscle, AICAR incubation with the same concentration did not affect PGC-1α expression in the rat soleus muscle (Fig. 5). AICAR treatment at the concentration of 0.5 mM is known to induce ~90% of the maximal physiologial effects of AICAR on glucose transport in the muscle (1, 7), suggesting that the concentration may virtually induce maximal activation of the AMPK signals in the physiologic condition. However, because this concentration of AMPK did not affect the expression of PGC-1α in soleus muscle, we further tested the effect of incubation with a 10-fold higher concentration (5.0 mM) of AICAR on PGC-1α in soleus muscle. However, we could not find any changes in PGC-1α expression in soleus muscle. These results may suggest that AMPK is not involved in the mechanism that induces the enhanced expression of PGC-1α in low-intensity running exercise. The AMPK pathway is also known to be uninvolved in the exercise-induced enhancement of glucose transport ac-

**Table 2. Effects of 18-h incubation with 0.5 mM AICAR and 4 mM caffeine on glycogen concentrations in rat epitrochlearis and soleus muscles**

<table>
<thead>
<tr>
<th>Muscle Glycogen Concentration</th>
<th>Control</th>
<th>AICAR</th>
<th>Caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epitrochlearis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preincubation</td>
<td>27.0 ± 4.2</td>
<td>25.8 ± 2.1</td>
<td>23.3 ± 16.8</td>
</tr>
<tr>
<td>Postincubation</td>
<td>25.6 ± 6.2</td>
<td>33.8 ± 7.6*</td>
<td>16.8 ± 4.5†</td>
</tr>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preincubation</td>
<td>13.3 ± 2.8</td>
<td>13.2 ± 1.5</td>
<td>14.1 ± 3.5</td>
</tr>
<tr>
<td>Postincubation</td>
<td>15.9 ± 2.8</td>
<td>12.7 ± 2.8</td>
<td>10.7 ± 2.1*</td>
</tr>
</tbody>
</table>

Values are means ± SD in μmol/g tissue. *P < 0.05, †P < 0.01: significantly different from values obtained in each preincubation group.
tivity in soleus muscles, because glucose transport activity in the muscle was not affected by the AICAR treatment (1, 5).

For the purpose of identifying possible candidates for the mediator of running-induced PGC-1α expression in rat soleus muscle, we observed the effect of caffeine incubation, which is known to release Ca^{2+} from SR (31), on PGC-1α protein expression in rat soleus muscle. We studied this effect because exposure to calcium ionophore, ionomycin, and caffeine increased the mitochondrial enzymes in the L6 myotubes (27, 28), suggesting that elevated cytosolic Ca^{2+} induces skeletal muscle mitochondrial biogenesis, which we hypothesized to be mediated by PGC-1α. Consequently, we found that exposure to caffeine in vitro increased the PGC-1α protein in rat soleus muscle (Fig. 5). These results may suggest that an increase in Ca^{2+} concentration in rat soleus muscle during exercise induces the enhanced expression of PGC-1α, which might induce mitochondrial biogenesis in the muscle, probably through calcium/calmodulin-dependent protein kinase IV (43).

Previously (39), we demonstrated that [Ca^{2+}]_{i} is chronically elevated by incubation with caffeine in rat skeletal muscle. In contrast, during exercise there are cyclical fluctuations of [Ca^{2+}]_{i}, such that [Ca^{2+}]_{i} is increased during contraction and reduced during relaxation. Therefore, the nonphysiological and chronic elevation in [Ca^{2+}]_{i} during the in vitro incubation with caffeine in the present investigation might not reflect the identical consequence that was invoked during exercise. Recently, Ojuka et al. (27) reported that the caffeine-induced elevation of [Ca^{2+}]_{i} in L6 myotubes increased mitochondrial proteins as well as PGC-1α and nuclear respiratory factors 1 and 2 (NRF-1, NRF-2) and mitochondrial transcriptional factor A (mtTFA), which was also observed in skeletal muscle after a single bout of exercise (4). Therefore, these observations may support the hypothesis that the increase in [Ca^{2+}]_{i} mediates the exercise-induced increase in PGC-1α and, consequently, in mitochondria in the skeletal muscle.

Because caffeine treatment in vitro also induced PGC-1α expression in the epitrochlearis muscle (Fig. 4), both AMPK activation and Ca^{2+} elevation in the muscle are suggested to induce PGC-1α expression after swimming exercise. It is interesting that, in terms of PGC-1α expression after exercise, the number of mechanisms differs between the two specific muscles (2, 26). It is possible that differences in fiber type may explain the different mechanisms related to PGC-1α expression after exercise. Previous studies (9) reported that the chronic effects of AICAR on glucose transport activity and GLUT4 content in rat skeletal muscle were present only in fast-twitch fiber-dominant muscles.

Eighteen-hour incubation with oxygenated culture medium did not affect the glycogen concentration in the epitrochlearis and soleus muscles of the control group of rats (Table 2), suggesting that the prolonged incubation in vitro may not cause metabolic disturbances that can be induced by hypoxia, which is known to reduce muscle glycogen concentration (10). In epitrochlearis muscle, adequate ATP and creatine phosphate levels were shown to be maintained during prolonged incubation in vitro (26). Our result might also indicate that the caffeine-induced decrease in glycogen concentration (Table 2) could be ascribed to enhanced glycogen phosphorylase activity induced by Ca^{2+} (14), not to hypoxia.

For the in vitro experiment of the present investigation, epitrochlearis and soleus muscles from relatively younger and smaller rats with body weights of 50–110 g were used for the in vitro experiments, because the diffusion of oxygen was shown to be secured in the in vivo experiments (12). Because the expression patterns of PGC-1α in epitrochlearis and soleus muscle after the low-intensity prolonged swimming and running exercises were not different between younger (body wt: 50–110 g) and older (body wt: 150–170 g) rats, it is plausible that the age-related effect is negligible in terms of the PGC-1α expression of skeletal muscle after single bouts of exercise.

PGC-1α is reported to bind to and coactivate the transcriptional function of NRF-1 on the promoter for mtTFA, a direct regulator of mitochondrial DNA replication/transcription (44). Because acute exercise and in vivo electrical stimulation are reported to increase NRF-1 (25) and mtTFA (11), respectively, these previous studies and the results obtained in the present investigation might suggest that some of the increase in mitochondrial protein that occurs after swimming and running exercise training is induced through the enhanced expression of PGC-1α protein. However, Bergeron et al. (6) suggested that the AMPK activation after β-guanidinopropionic acid feeding may directly enhance the NRF-1 binding activity and the expression of respiratory proteins. As we have described, several plausible hypotheses may explain physical exercise-induced mitochondrial biogenesis. However, it is still not precisely known whether the mechanism of the exercise training-induced increase in skeletal muscle mitochondrial oxidative enzymes involves PGC-1α.

Because the present investigation demonstrated that AICAR and caffeine increased PGC-1α protein content, the increased GLUT4 content after chronic AICAR (9, 5, 15, 42) and caffeine (29) treatment might be attributed in part to PGC-1α-induced activation of myocyte enhancer factor-2, which has been suggested to activate GLUT4 transcription (24). A higher expression of PGC-1α was also found in slow-twitch fiber-dominant muscles, such as the soleus muscle and the red region of the gastrocnemius muscle (Fig. 3), which possess more mitochondria and GLUT4 proteins. Furthermore, the similarity of the site-specific expression of PGC-1α to that of GLUT4 and mitochondrial enzymes (19, 36) might suggest the involvement of exercise-enhanced PGC-1α expression in the subsequent exercise training-induced increase of GLUT4 and mitochondrial enzymes that has been suggested to be coexpressed by the same mechanisms (35). However, it remains unknown whether PGC-1α is involved in the exercise training-induced increase in GLUT4 in skeletal muscle.

In conclusion, the present investigation demonstrated that acute bouts of low-intensity prolonged swimming and running exercise increased the PGC-1α protein content in the rat skeletal muscles that were recruited during each exercise. Furthermore, the results obtained in the present investigation suggest that exercise-induced PGC-1α expression in rat skeletal muscle may be mediated by at least two exercise-induced biochemical factors, AMPK activation and the elevation of Ca^{2+}. Furthermore, the results suggest that the number of factors involved (both AMPK and Ca^{2+} or Ca^{2+} only) depends on the specific muscle, and possibly on the muscle fiber type that is dominant.

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REFERENCES

40. Terada S, Yokozeki T, Kawanaka K, Ogawa K, Higuchi M, Ezaki O, and Tabata I. Effects of high-intensity swimming training on GLUT-4