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ORIGINAL PAPER

The effects of β-adrenergic stimulation and exercise on NR4A3 protein expression in rat skeletal muscle

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Abstract β-Adrenergic stimulation and exercise up-regulate the mRNA expression of nuclear receptor NR4A3, which is involved in the regulation of glucose and fatty acid utilization genes in skeletal muscle. The objective of our study was to examine the effects of β-adrenergic stimulation and exercise on the expression of NR4A3 protein in rat skeletal muscle. A single subcutaneous injection of clenbuterol, which is a β2-adrenergic receptor (β2-AR) agonist, increased NR4A3 mRNA and protein expression in the fast-twitch glycolytic triceps muscle. On the other hand, an acute 3-h session of either treadmill running or swimming did not increase the NR4A3 protein level in the exercised muscle, although both treadmill running and swimming increased NR4A3 mRNA. Finally, loss of postural contractile activity because of hindlimb immobilization reduced NR4A3 mRNA and protein in the slow-twitch oxidative soleus muscle. These results suggest that: β-adrenergic stimulation up-regulates not only NR4A3 mRNA but also NR4A3 protein in fast-twitch glycolytic muscle; exercise may increase NR4A3 mRNA but not NR4A3 protein in skeletal muscle; and local postural contractile activity plays a crucial role in maintaining NR4A3 protein expression level in postural muscle.

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Introduction

Skeletal muscle is a major mass metabolic tissue that accounts for approximately 40% of total body weight. This peripheral lean tissue is the primary site for fatty acid oxidation. Furthermore, this tissue is the main target of insulin-stimulated glucose uptake, and muscular glucose uptake accounts for approximately 80% of glucose disposal [1]. Therefore, the ability of skeletal muscle to utilize glucose and fatty acids is involved in the pathophysiological progression of metabolic diseases such as dyslipidemia, diabetes, and obesity. Several lines of evidence have demonstrated that skeletal muscle is a target of β-adrenergic signaling, and β-adrenergic receptor agonists increase fatty acid utilization in skeletal muscle [2–4]. Furthermore, physical exercise increases the ability of skeletal muscle to utilize glucose and fatty acid, leading to the prevention of metabolic diseases [5–7]. However, the molecular regulatory mechanism(s) accounting for these effects of β-adrenergic stimulation or exercise are not yet fully understood.

The NR4A orphan nuclear receptor subfamily consists of three well-conserved members, NR4A1, NR4A2, and NR4A3, known in mice as Nur77, Nurr1, and NOR1, respectively. Although the potential roles of NR4A receptors in metabolism have not been well explored, previous studies have demonstrated that attenuation of NR4A3 gene expression reduced the expression of genes associated with glucose and fatty acid utilization (e.g., peroxisome proliferator-activated receptor γ coactivator (PGC)-1α/β, lipin-1α, etc.) in a skeletal muscle cell line

[8, 9]. Furthermore, Fu et al. [10] reported that gene expression of NR4A3 was reduced in the skeletal muscle of diabetic and insulin-resistant animals. They also showed that hyperexpression of NR4A3 increased the ability of insulin to stimulate glucose transport and glucose transporter type 4 (GLUT4) translocation. Thus, NR4A3 gene expression is thought to be involved in the regulation of genes that control glucose and fatty acid utilization in skeletal muscle.

NR4A3 mRNA expression is increased by β-adrenergic receptor agonists in skeletal muscle [8, 9]. Previous studies including ours showed that exercise increased NR4A3 mRNA expression in skeletal muscle [11, 12]; ours also showed that exercise activates adenosine monophosphate (AMP)-activated protein kinase (AMPK) in the exercising muscles and that pharmacological activation of AMPK increases NR4A3 mRNA expression in these muscles [11]. This result suggests that increased NR4A3 mRNA expression in exercised muscles is possibly mediated by AMPK activation, at least in part.

The above-mentioned results suggest that increased gene expression of NR4A3 may account for the effects of β-adrenergic stimulation or exercise on glucose and fatty acid utilization in skeletal muscle. However, the effects of β-adrenergic stimulation or exercise on the expression of NR4A3 protein in skeletal muscle are still unknown. Therefore, in this study, using experimental animals, we examined whether β-adrenergic stimulation, exercise, or pharmacological activation of AMPK increases not only NR4A3 mRNA but also NR4A3 protein expression in rat skeletal muscle. In addition, we tested whether local postural contractile activity regulates NR4A3 protein expression in postural muscle using a one-legged immobilization model.

Materials and methods

Treatment of animals

This research was approved by the Animal Studies Committee of Niigata University of Health and Welfare. Male Wistar rats were obtained from CLEA Japan (Tokyo, Japan). Animals were housed in an animal room maintained at 20–22°C with a 12:12-h light–dark cycle and fed a standard rodent chow diet and water ad libitum.

β2-Adrenergic receptor agonist clenbuterol administration

Rats (100–120 g) were randomly divided into a time-matched saline control (Saline control) group ($n = 5$–6 for each time point) or a β2-adrenergic receptor (β2-AR) agonist clenbuterol (Clenbuterol) group ($n = 5$–6 for each time point). All rats were fed ad libitum until clenbuterol injection. Clenbuterol group rats received a single subcutaneous injection of clenbuterol (100 μg/100 g body wt). Saline control group rats received an equivalent volume of saline. Rats were anesthetized with sodium pentobarbital (5 mg/100 g body wt) 4, 8, or 12 h after clenbuterol injection, and the soleus and triceps muscles were removed and clamp-frozen in liquid nitrogen for measurement of NR4A3 mRNA and protein expression. The clenbuterol group rats were time-matched with the saline control group rats, with the tissues of the control rats being collected at the same times as those of the clenbuterol group rats.

A single bout of exercise

Two days before the experiment, all rats were acclimated to treadmill running or swimming for 10 min. Rats (100–120 g) were randomly divided into a time-matched resting control (Resting control) group ($n = 5$–8 for each time point), a treadmill running (Acute treadmill) group ($n = 8$ for each time point), or a swimming (Acute swimming) group ($n = 5$–8 for each time point). All rats were fed ad libitum until the treadmill or swimming group started exercise. When the treadmill or swimming group rats started exercise, the resting control group rats started fasting. Rats in the acute treadmill group ran on a motorized treadmill up a 15% gradient at 9 m/min for 3 h [11]. Rats in the acute swimming group swam for 3 h without a weight [11, 13]. The water temperature was maintained at 35°C during the swimming. Animals were returned to their cages after completion of treadmill running or swimming and kept fasted.

Following the acute treadmill running exercise, rats were anesthetized with pentobarbital sodium (5 mg/100 g body wt) 0, 4, or 8 h after completion of the treadmill running, and soleus muscle was dissected and clamp-frozen in liquid nitrogen. NR4A3 mRNA was measured in soleus muscles dissected 0 h after completion of running. NR4A3 protein was measured in soleus muscles dissected 0, 4, and 8 h after completion of running. Soleus muscle was chosen because we previously demonstrated that soleus muscle was sufficiently recruited during our treadmill running procedure and NR4A3 mRNA expression in soleus muscle was increased by our treadmill running procedure [11].

Following the acute swimming exercise, rats were anesthetized with pentobarbital sodium (5 mg/100 g body wt) 0, 4, or 8 h after completion of the exercise, and triceps muscle was dissected and clamp-frozen in liquid nitrogen. NR4A3 mRNA was measured in triceps muscles dissected 0 h after completion of swimming. NR4A3 protein was measured in triceps muscles dissected 0, 4, and 8 h after completion of swimming. Triceps muscle was chosen

because we previously demonstrated that triceps muscle was sufficiently recruited during our swimming procedure and NR4A3 mRNA expression in triceps muscle was increased by our swimming procedure [11].

The treadmill and swimming groups were time-matched with the resting control group, with the tissues of the resting control group rats being collected at the same time as those of the exercised rats.

Chronic exercise training

For the first set of chronic exercise training experiments, rats (100–120 g) were randomly assigned to either an untrained-sedentary control (Sedentary control) group ($n = 6$) or a treadmill-running training (Treadmill training) group ($n = 6$). The treadmill training rats were trained for a total of 3 weeks by having them run on a motorized treadmill with a 15% gradient. The speed and duration of their running were 10 m/min and 3 h/day, respectively. All rats were maintained in individual cages and fed a powdered standard rodent chow diet and water ad libitum. All rats were fasted for ~12 h before the muscle sampling. Between 0 and 4 pm (~21 h after the last treadmill running bout), the rats were anesthetized with pentobarbital sodium (5 mg/100 g body wt). Then, their soleus muscle was dissected and clamp-frozen in liquid nitrogen for subsequent measurement of NR4A3, PGC-1α, and cytochrome C oxidase subunit I (COX I) protein levels. Soleus muscle was chosen because this muscle is sufficiently recruited during treadmill running [11].

For the second set of chronic exercise training experiments, rats (100–120 g) were randomly assigned to either an untrained-sedentary control (Sedentary control) group ($n = 7$) or a voluntary wheel-running training (Voluntary training) group ($n = 7$). Sedentary control rats were housed in individual cages and fed a powdered standard rodent chow diet and water ad libitum. The voluntary training rats were housed in individual cages with mesh wire running wheels (1.07-m circumference, 10-cm-wide running surface; Shinano Instruments, Tokyo, Japan) for 3 weeks. Between 6 am and 9 am (~12 h before the muscle sampling), voluntary wheel training rats were denied access to the running wheel and food was removed from their cages. Sedentary control rats were also made to fast for ~12 h before the muscle sampling. Between 6 and 9 pm the rats were anesthetized with pentobarbital sodium (5 mg/100 g body wt). Then, their triceps muscle was dissected and clamp-frozen in liquid nitrogen for subsequent measurement of NR4A3, PGC-1α, and COX I protein levels. Triceps muscle was chosen because this muscle is sufficiently recruited during voluntary wheel running [14].

Pharmacological AMPK activator AICAR administration

Rats (100–120 g) were randomly divided into a time-matched saline control (Saline control) group ($n = 6$–7 for each time point) or an AICAR administration (AICAR) group ($n = 6$–7 for each time point). AICAR group rats were given daily subcutaneous injections, between 5 and 7 pm, of AICAR (50 mg/100 g body wt) for 7 days. Saline control group rats were given daily subcutaneous injections of saline vehicle. Rats were anesthetized with sodium pentobarbital (5 mg/100 g body wt) 1 and 24 h after the last AICAR injection, and the soleus and triceps muscles were removed and clamp-frozen in liquid nitrogen for measurement of NR4A3 mRNA, NR4A3 protein, and PGC-1α protein expression. The AICAR group rats were time-matched with the saline control group rats, with the tissues of the control rats being collected at the same times as those of the AICAR group rats.

Immobilization

Rats (100–120 g) ($n = 8$) were subjected to unilateral hindlimb immobilization. A plaster cast (Castlight, Alcare, Tokyo, Japan) was applied to the left hindlimb of each rat without anesthetization. The leg was immobilized in the planter flexion position. Results from pilot studies (data not shown) indicated that unilateral immobilization had no effect on basal and insulin (50 μU/mL)-stimulated glucose transport activity in soleus muscle from the contralateral noncasted leg. Consequently, the contralateral hindlimb served as the control in our experiment. After casting, rats were housed individually. Immobilization was imposed for 6 h. All rats were maintained in individual cages and fed a standard rodent chow diet and water ad libitum until the muscle sampling. For muscle sampling, casts were removed under pentobarbital sodium anesthesia (5 mg/100 g body wt), and soleus muscles from both the immobilized and contralateral hindlimb were dissected and either clamp-frozen in liquid nitrogen for subsequent measurement of NR4A3 mRNA and protein, or used for the measurement of 2-deoxyglucose (2DG) uptake as described below.

Western blot analysis

Triceps or soleus muscles were homogenized in ice-cold buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 2 mM PMSF, aprotinin (10 μg/mL), leupeptin (10 μg/mL), and pepstatin (5 μg/mL) [15]. The homogenates were then rotated end-over-end at 4°C for

60 min and centrifuged at 4,000*g* for 30 min at 4°C. Aliquots of the supernatants were treated with 2× Laemmli sample buffer containing 100 mM dithiothreitol. For measurement of NR4A3 and COX I protein levels, the samples were run on 10% SDS-PAGE; for measurement of PGC-1α protein level, the samples were run on 7% SDS-PAGE. The resolved proteins were then transferred to PVDF membranes. The membranes were incubated with NR4A3 antibody (Perceus Proteomics, Tokyo, Japan) that was used in a previous study [16], PGC-1α antibody (Calbiochem, San Diego, CA, USA), and COX I antibody (Invitrogen, Eugene, OR, USA). The membranes were further incubated with HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or HRP-conjugated anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK). Antibody bound protein was visualized by enhanced chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK) with the intensity of the bands being quantified by use of densitometry.

Real-time PCR analysis for measurement of mRNA expression

Muscles were homogenized in 600 μL RNAgents (Promega, Madison, WI, USA) by use of a polytron homogenizer (Polytron PT2100, Kinematica). RNA was isolated according to the manufacturer's instructions. The purified RNA was reverse-transcribed with a reverse transcription system (Promega). Measurement of reverse-transcribed cDNA was carried out with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with primers to NR4A3 (forward AGACAAGAGA CGTCGAAATCGAT; reverse CTTCACCATCCCGAC ACTGA) and GAPDH (forward GACAACTTTGGCA TCGTGAA; reverse ATGCAGGGATGATGTTCTGG). Fluorescence was monitored during PCR by use of an ABI7300 Sequence Detection System (Applied Biosystems). PCR data were then normalized by GAPDH expression.

Measurement of 2-deoxyglucose uptake

The rate of muscle glucose transport was determined by the method described by Koshinaka et al [13]. The soleus muscles were incubated with shaking for 20 min at 30°C in 3 mL oxygenated Krebs–Hensleit buffer (KHB) containing 40 mM mannitol and 0.1% RIA grade bovine serum albumin (BSA), in the absence or presence of purified human insulin (50 μU/mL). After the 20-min incubation described above, the soleus muscles were incubated for 20 min at 30°C in 3 mL KHB containing 8 mM 2DG, 32 mM mannitol, and 0.1% BSA without or with insulin at the same concentration as in the initial 20-min incubation described above. The flasks were gassed continuously with 95% O_2–5% CO_2 during incubation. After incubation, the muscles were blotted, clamp-frozen in liquid nitrogen, and used for fluorimetric measurement of 2-deoxyglucose-6-phosphate (2DG6P) by the method described by Passonneau et al [17].

Statistical analysis

Data are expressed as mean ± SE. Differences were determined by use of an unpaired Student's *t* test. Differences between groups were considered statistically significant when $P < 0.05$.

Results

Effect of treatment with the β2-AR agonist clenbuterol

To evaluate the effect of β2-AR stimulation on NR4A3 gene expression in skeletal muscle, we measured NR4A3 mRNA and protein expression in slow-twitch oxidative (soleus) and fast-twitch glycolytic (triceps) muscle of rats treated with the β2-AR agonist clenbuterol. As shown in Fig. 1a, 2.5-fold significant increases in NR4A3 mRNA were observed in soleus muscle 4 h after a single subcutaneous injection of clenbuterol compared with a time-matched saline control ($P < 0.05$). The elevated expression of NR4A3 mRNA in soleus muscle returned to the saline control level 12 h after clenbuterol injection (Fig. 1a). On the other hand, no significant increase in NR4A3 protein was observed in soleus muscle 4, 8, and 12 h after clenbuterol injection (Fig. 1b).

As shown in Fig. 2a, NR4A3 mRNA expression was increased 33.3-fold in triceps muscle 4 h after clenbuterol injection compared with time-matched saline control ($P < 0.05$). NR4A3 mRNA in triceps muscle tended to be increased 8 h after clenbuterol injection ($P = 0.07$). Increased expression of NR4A mRNA in triceps muscle returned to the saline control level 12 h after clenbuterol injection (Fig. 2a). The NR4A3 protein level tended to be increased in triceps muscle 4 h after clenbuterol injection ($P = 0.09$, Fig. 2b), and NR4A3 protein was 37 and 18% increased 8 and 12 h after clenbuterol injection, respectively, compared with time-matched saline controls ($P < 0.05$, Fig. 2b).

Effect of a single bout of exercise

To examine the effect of a single bout of exercise on NR4A3 protein expression in skeletal muscle, we subjected rats to an acute 3-h treadmill running session or a 3-h swimming session. Soleus but not triceps muscle is

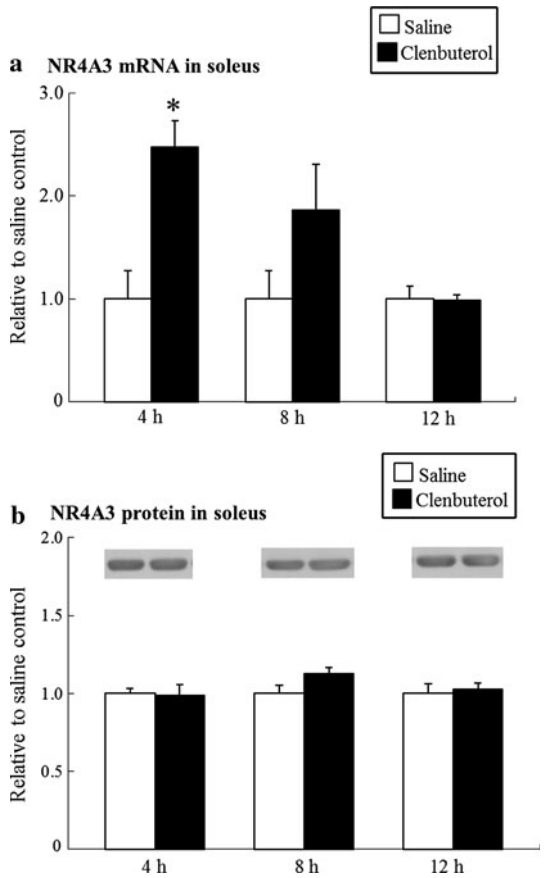

Fig. 1 NR4A3 mRNA (**a**) and protein (**b**) in rat soleus muscle after treatment with the β2-AR agonist clenbuterol. Rats were divided into a saline control (*Saline*) group or a clenbuterol administration (*Clenbuterol*) group. Clenbuterol group rats received a single subcutaneous injection of clenbuterol (100 μg/100 g body wt). Saline control group rats received an equivalent volume of saline. Soleus muscle was removed 4, 8, or 12 h after clenbuterol injection for measurement of NR4A3 mRNA and protein expression. **a** Relative amounts of NR4A3 mRNA are expressed as mean ± SE ($n = 5$–6). **b** Relative amounts of NR4A3 protein are expressed as mean ± SE ($n = 5$–6). Representative western blots are shown at the *top* of the figure. *$P < 0.05$ versus Saline control

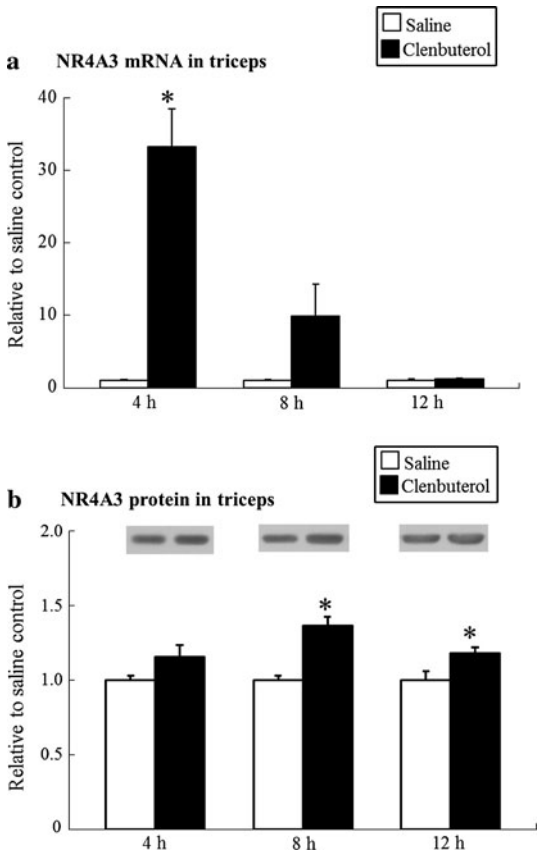

Fig. 2 NR4A3 mRNA (**a**) and protein (**b**) in rat triceps muscle after treatment with the β2-AR agonist clenbuterol. Rats were divided into a saline control (*Saline*) group or a clenbuterol administration (*Clenbuterol*) group. Clenbuterol group rats received a single subcutaneous injection of clenbuterol (100 μg/100 g body wt). Saline control group rats received an equivalent volume of saline. Triceps muscle was removed 4, 8, or 12 h after clenbuterol injection for measurement of NR4A3 mRNA and protein expression. **a** Relative amounts of NR4A3 mRNA are expressed as mean ± SE ($n = 5$–6). **b** Relative amounts of NR4A3 protein are expressed as mean ± SE ($n = 5$–6). The mean value in the saline control muscle was set at 1.0. Representative western blots are shown at the *top* of the figure. *$P < 0.05$ versus Saline control

sufficiently recruited during treadmill running, whereas triceps but not soleus muscle is sufficiently recruited during swimming [11]. Therefore, we measured NR4A3 protein in soleus muscle of rats subjected to treadmill running and in triceps muscle of rats subjected to swimming. As shown in Fig. 3, although a single bout of treadmill running increased NR4A3 mRNA in soleus muscle 23.3-fold immediately after the cessation of exercise, treadmill running did not change NR4A3 protein level in soleus muscle after the cessation of exercise compared with time-matched resting controls. As shown in Fig. 4, although a single bout of swimming increased NR4A3 mRNA in triceps muscle 7.4-fold immediately after the cessation of exercise, no significant increase in NR4A3 protein was observed in triceps muscle after a single bout of swimming.

Effect of chronic exercise training

To examine the possibility that NR4A3 protein expression was increased by chronic exercise training, we subjected rats to 3 weeks of treadmill or voluntary running training. As described above, soleus muscle is sufficiently recruited during treadmill running [11], and triceps muscle is sufficiently recruited during voluntary running [14]. Therefore, we measured NR4A3 protein in soleus muscle of rats subjected to treadmill running training and in triceps muscle of rats subjected to voluntary running training. As shown in Table 1, the NR4A3 protein level in soleus muscle was not increased after 3 weeks of treadmill running training. On the other hand, the protein expression of PGC-1α and COX I,

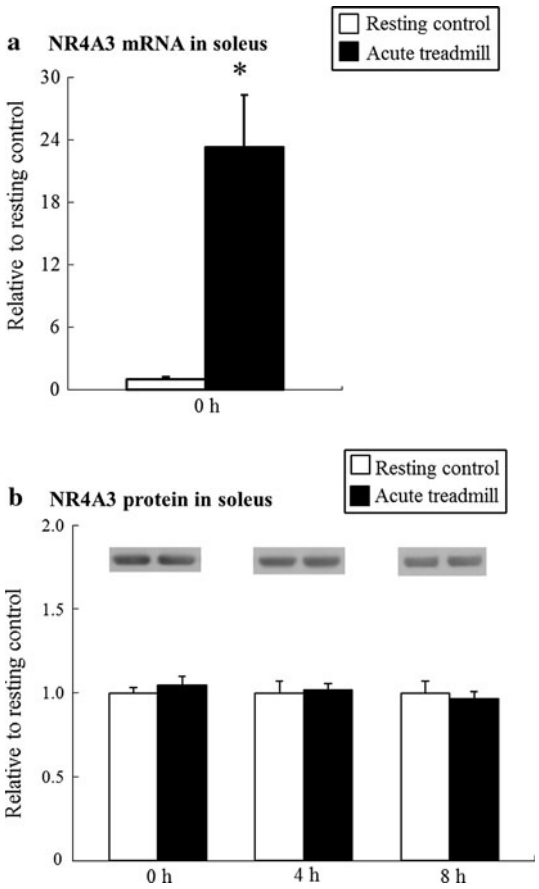

Fig. 3 NR4A3 mRNA (**a**) and protein (**b**) in rat soleus muscle after cessation of a single bout of treadmill running. Rats were divided into a resting control (*Resting control*) group or a treadmill running (*Acute treadmill*) group. Acute treadmill rats were subjected to an acute bout of treadmill running up a 15% gradient at 9 m/min for 3 h. Soleus muscle was removed 0, 4, or 8 h after acute treadmill running for measurement of NR4A3 mRNA and protein expression. **a** NR4A3 mRNA was measured 0 h after acute treadmill running. Relative amounts of NR4A3 mRNA are expressed as mean ± SE ($n = 8$). **b** NR4A3 protein was measured 0, 4, and 8 h after acute treadmill running. Relative amounts of NR4A3 protein are expressed as mean ± SE ($n = 8$). Representative western blots are shown at the *top* of the figure. *$P < 0.05$ versus Rest control

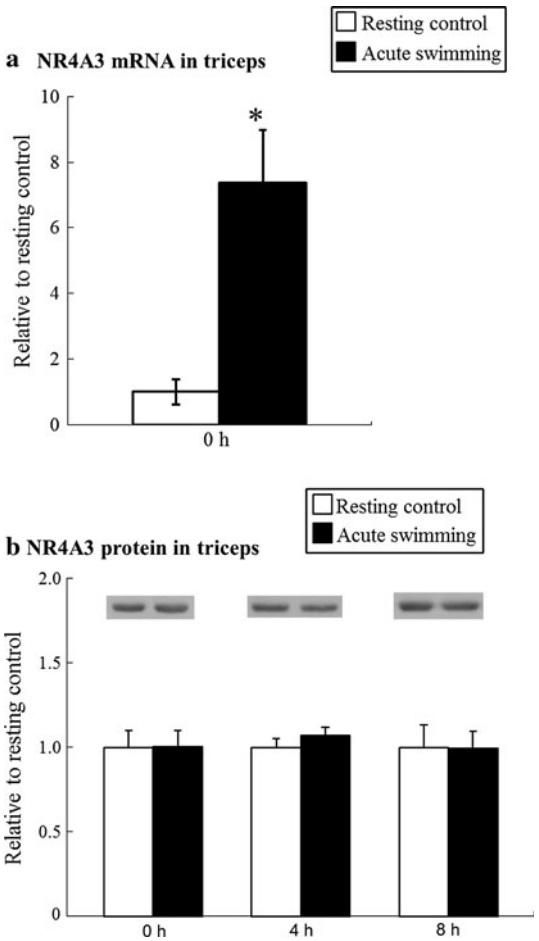

Fig. 4 NR4A3 mRNA (**a**) and protein (**b**) in rat triceps muscle after cessation of a single bout of swimming. Rats were divided into a resting control (*Resting control*) group or a swimming (*Acute swimming*) group. Acute swimming rats were subjected to an acute 3-h-long bout of swimming without a weight. Triceps muscle was removed 0, 4, or 8 h after acute swimming for measurement of NR4A3 mRNA and protein expression. **a** NR4A3 mRNA was measured 0 h after acute swimming. Relative amounts of NR4A3 mRNA are expressed as mean ± SE ($n = 8$). **b** NR4A3 mRNA was measured 0, 4, and 8 h after acute swimming. Relative amounts of NR4A3 protein are expressed as mean ± SE ($n = 5$–8). Representative western blots are shown at the *top* of the figure. *$P < 0.05$ versus Rest control

which are known to be a master regulator of mitochondrial biogenesis and a marker of mitochondrial enzymes, were increased by 32 and 19%, respectively, in soleus muscle of treadmill runners (Table 1). Moreover, no significant increase in NR4A3 protein was observed in triceps muscle after 3 weeks of voluntary running training, whereas PGC-1α and COX I were increased by 49 and 58%, respectively, in triceps muscle of voluntary runners (Table 2).

Effect of chronic AMPK activation

To examine the effects of chronic AMPK activation, we gave rats daily subcutaneous injections of AICAR for 7 days. In order to distinguish the acute response from chronic adaptations to the AICAR treatment, soleus and triceps muscles were dissected 1 and 24 h after the final AICAR injection. Daily AICAR injection significantly increased NR4A3 mRNA in soleus muscle 1 h ($P < 0.05$) but not 24 h after the final injection (1 h: 1.00 ± 0.10 ($n = 7$) for saline injection and 1.81 ± 0.20 ($n = 7$) for AICAR injection, 24 h: 1.00 ± 0.09 ($n = 6$) for saline injection, and 0.89 ± 0.05 ($n = 6$) for AICAR injection). Furthermore, daily AICAR injection significantly increased NR4A3 mRNA in triceps muscle 1 h ($P < 0.05$) but not 24 h after the final injection (1 h: 1.00 ± 0.20 ($n = 7$) for

Table 1 The effects of 3 weeks of treadmill running training on NR4A3, PGC-1α, and COX I protein levels in rat soleus muscles

	Sedentary	Treadmill running
NR4A3	1.00 ± 0.04	0.90 ± 0.04
PGC-1α	1.00 ± 0.06	1.32 ± 0.13*
COX I	1.00 ± 0.08	1.19 ± 0.03*

Relative protein levels are expressed as mean ± SE for 6 muscles/group

Significant differences from sedentary control, * $P < 0.05$

Table 2 The effects of 3 weeks of voluntary running training on NR4A3, PGC-1α, and COX I protein levels in rat triceps muscles

	Sedentary	Voluntary running
NR4A3	1.00 ± 0.05	1.02 ± 0.09
PGC-1α	1.00 ± 0.15	1.49 ± 0.15*
COX I	1.00 ± 0.15	1.58 ± 0.20*

Relative protein levels are expressed as mean ± SE for 6–7 muscles/group

Significant differences from sedentary control, * $P < 0.05$

Table 3 The effects of chronic AICAR treatment on NR4A3 and PGC-1α protein levels in rat soleus muscles

	Saline	AICAR
NR4A3	1.00 ± 0.04	0.99 ± 0.03
PGC-1α	1.00 ± 0.03	1.15 ± 0.05*

Rats were given daily subcutaneous AICAR injections for 7 days. Soleus muscles were removed 24 h after the final AICAR injection. Relative protein levels are expressed as mean ± SE for 6 muscles/group

Significant differences from saline control, * $P < 0.05$

saline injection and 2.74 ± 0.16 ($n = 7$) for AICAR injection, 24 h: 1.00 ± 0.19 ($n = 6$) for saline injection and 0.58 ± 0.06 ($n = 5$) for AICAR injection). Thus, AMPK activation acutely increases NR4A3 mRNA in skeletal muscles.

On the other hand, daily AICAR injection for 7 days did not increase NR4A3 protein in soleus muscle 1 and 24 h after the final injection (1 h: 1.00 ± 0.05 ($n = 7$) for saline injection and 0.95 ± 0.02 ($n = 7$) for AICAR injection, 24 h; Table 3). Moreover, daily AICAR injection did not increase NR4A3 protein in triceps muscle 1 and 24 h after the final injection (1 h: 1.00 ± 0.08 ($n = 7$) for saline injection and 1.00 ± 0.04 ($n = 7$) for AICAR injection, 24 h; Table 4). In contrast, as shown in Tables 3 and 4, daily AICAR injection significantly increased PGC-1α protein in soleus and triceps muscles 24 h after the final injection ($P < 0.05$).

Table 4 The effects of chronic AICAR treatment on NR4A3 and PGC-1α protein levels in rat triceps muscles

	Saline	AICAR
NR4A3	1.00 ± 0.05	0.95 ± 0.03
PGC-1α	1.00 ± 0.17	1.68 ± 0.17*

Rats were given daily subcutaneous AICAR injections for 7 days. Triceps muscles were removed 24 h after the final AICAR injection. Relative protein levels are expressed as means ± SE for 6 muscles/group

Significant differences from saline control, * $P < 0.05$

The difference between muscle types

Using resting sedentary rats, we examined the difference in NR4A3 mRNA and protein level between slow-twitch oxidative (soleus) and fast-twitch glycolytic (triceps) muscle. As shown in Fig. 5, NR4A3 mRNA and protein levels in soleus muscle were significantly higher than those in triceps muscle ($P < 0.05$).

Effect of immobilization

To examine the role of postural contractile activity, we subjected rats to unilateral hindlimb immobilization. As shown in Fig. 6, after 6 h of immobilization, NR4A3 mRNA and protein expression were reduced in immobilized soleus muscle by 30 and 27%, respectively, relative to contralateral non-immobilized control muscles ($P < 0.05$). Furthermore, we examined the effect of 6 h immobilization on glucose transport activity in soleus muscle. Basal (without insulin) and insulin (50 μU/mL)-stimulated glucose transport in the immobilized soleus muscle were reduced by 65 and 61%, respectively, compared with the contralateral non-immobilized muscle (basal: 1.41 ± 0.14 μmol/g muscle/20 min ($n = 5$) for the contralateral control muscle and 0.50 ± 0.10 μmol/g muscle/20 min ($n = 5$) for the immobilized muscle, insulin: 4.73 ± 0.17 μmol/g muscle/20 min ($n = 6$) for the contralateral control muscle and 1.86 ± 0.07 μmol/g muscle/20 min ($n = 6$) for the immobilized muscle) ($P < 0.05$).

Discussion

The most important results in this study were that, in rats:

1. a single subcutaneous injection of clenbuterol, a β2-AR agonist, increased NR4A3 mRNA and protein expression in the fast-twitch glycolytic triceps muscle;
2. neither an acute bout of treadmill running nor one of swimming increased NR4A3 protein expression in exercised muscle in which the NR4A3 mRNA level was elevated;

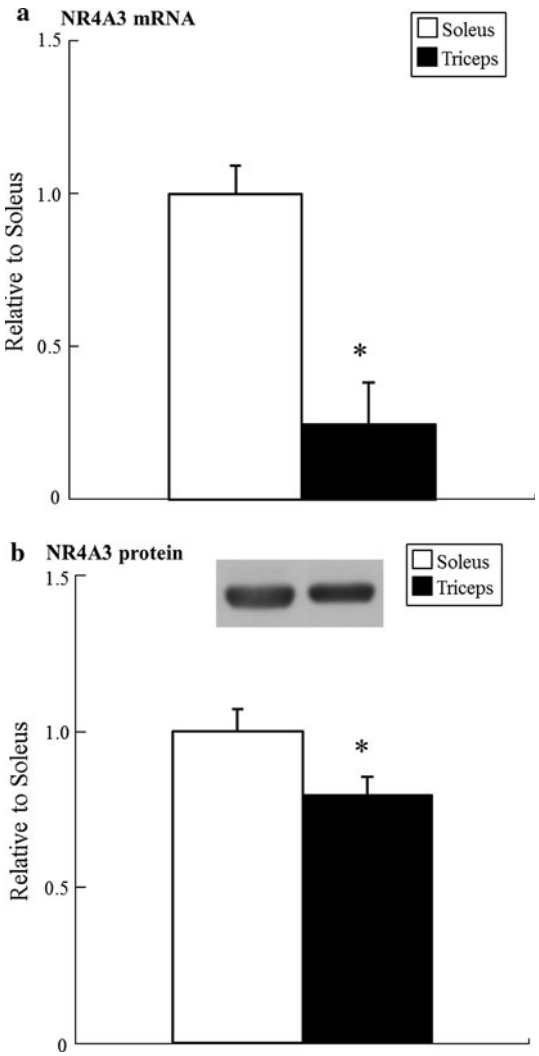

Fig. 5 NR4A3 mRNA (**a**) and protein (**b**) in rat slow-twitch oxidative soleus and fast-twitch glycolytic triceps muscles. **a** Relative amounts of NR4A3 mRNA are expressed as mean ± SE ($n = 4$). The mean value in the soleus muscle was set at 1.0. **b** Relative amounts of NR4A3 protein are expressed as mean ± SE ($n = 6$). The mean value in the soleus muscle was set at 1.0. Representative western blots are shown at the *top* of the figure. $^*P < 0.05$ versus soleus muscle

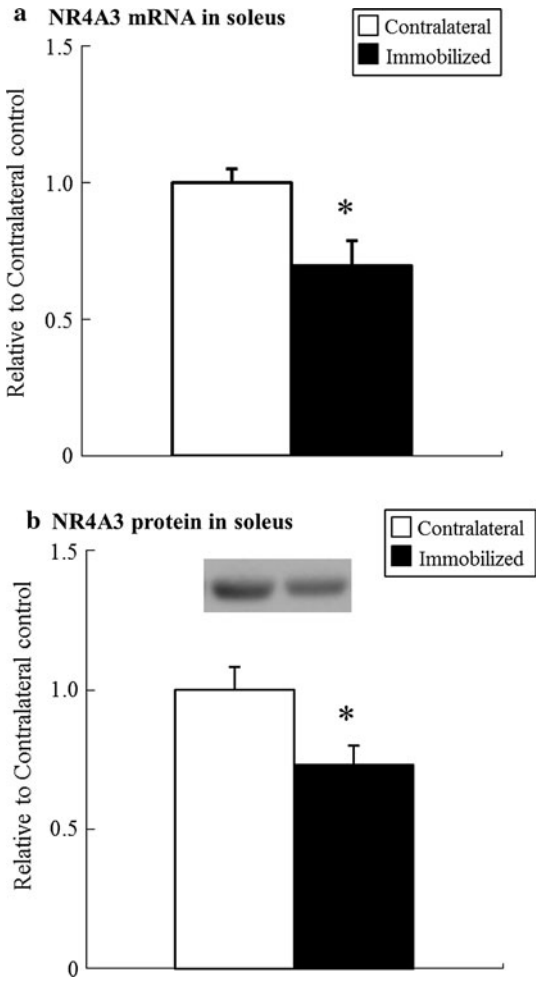

Fig. 6 NR4A3 mRNA (**a**) and protein (**b**) in rat soleus muscles after 6 h of hindlimb immobilization. **a** Relative amounts of NR4A 3mRNA are expressed as mean ± SE ($n = 8$). The mean value in the contralateral non-immobilized muscle was set at 1.0. **b** Relative amounts of NR4A3 protein are expressed as mean ± SE ($n = 8$). The mean value in the contralateral non-immobilized muscle was set at 1.0. Representative western blots are shown at the *top* of the figure. $^*P < 0.05$ versus contralateral non-immobilized muscle

3. chronic exercise training did not increase muscle NR4A3 protein expression;
4. daily chronic injection of AICAR, a pharmacological AMPK activator, did not increase muscle NR4A3 protein expression; and
5. loss of postural contractile activity due to hindlimb immobilization reduced NR4A3 mRNA and protein expression in the slow-twitch oxidative soleus muscle.

Skeletal muscle is a target of β-adrenergic signaling. Furthermore, a variety of studies have demonstrated that acute and chronic administration of a β2-AR agonist modulates energy expenditure, lipolysis, and fatty acid utilization in skeletal muscles [2–4]. However, the molecular mechanisms responsible for these effects of β2-AR agonists in skeletal muscle are currently unclear. In this study, NR4A3 mRNA expression was increased in both the slow-twitch oxidative soleus and the fast-twitch glycolytic triceps muscle of rats 4 h after treatment with the β2-AR-specific agonist clenbuterol (Figs. 1a, 2a). This is very consistent with the observation that treatment with the β2-AR agonist formoterol induces the expression of NR4A3 mRNA in murine skeletal muscle [8, 9]. Furthermore, in our study, we found that NR4A3 protein expression was also significantly and transiently increased in the triceps muscle 8–12 h after treatment with clenbuterol (Fig. 2b). To our knowledge, this is the first study to demonstrate that β2-AR stimulation increases the protein expression level of NR4A3 in rodent skeletal muscles.

Because it has been demonstrated that NR4A3 is implicated in the regulation of genes that control lipid metabolism in skeletal muscle [8, 9], it is possible that β2-AR stimulation up-regulates skeletal muscle metabolism via an increase in NR4A3 protein.

In this study it seems that treatment with the β2-AR agonist increases NR4A3 mRNA and protein to a much greater extent in the fast-twitch glycolytic triceps than in the slow-twitch soleus muscle (Figs. 1, 2). This result seems to be conflict with the evidence that the density of β2-AR in slow-twitch muscle fiber is 2–3-fold higher than in fast-twitch muscle fiber [18]. Currently, the reason for this conflict is not clear. However, higher density of β2-AR in the soleus muscle may result in a higher average 24 h level of catecholamine action in this muscle compared with the triceps muscle in the absence of any treatment, leading to a greater NR4A3 expression level, as shown in Fig. 5. Therefore, it may be possible that NR4A3 mRNA and protein expression reach near saturation level and are less responsive to β2-AR stimulation in the soleus muscle. Another possibility may be that greater increases in β2-AR stimulation are required to further increase NR4A3 expression in the soleus muscle.

According to our previous study, the soleus muscle but not the triceps muscle is recruited during an acute 3-h session of treadmill running (at 9 m/min up a 15% gradient for 3 h). Conversely, the triceps but not the soleus muscle is recruited during an acute 3-h session of swimming without weight [11]. Furthermore, in this previous study, we showed that NR4A3 mRNA expression was increased in muscles recruited during exercise. In agreement with these results, we observed that both an acute 3-h of treadmill running and swimming increased NR4A3 mRNA in the exercised muscles (Figs. 3a and 4a, respectively). Because exercise increases the ability of skeletal muscle to utilize glucose and fatty acids [5–7], we hypothesized that exercise increases this ability in exercising muscle via increased NR4A3 gene expression. However, in this study neither acute treadmill running nor swimming increased NR4A3 protein expression in muscles recruited during exercise (Figs. 3b and 4b, respectively). Furthermore, neither 3 weeks of chronic treadmill nor voluntary running training increased the NR4A3 protein level in exercised muscles, although both types of training seem to increase the ability of muscle to utilize glucose and fatty acids via increased PGC-1α and COX I, which are known to be a master mediator of mitochondrial biogenesis and a marker of mitochondrial enzymes, respectively (Tables 1 and 2). Thus, our previous and current results suggest the possibility that exercise promotes pretranslational processes for the increase in NR4A3 protein in exercised muscles, but posttranslational processes are not promoted in exercised muscles in which the NR4A3 mRNA is increased. Because increased gene expression and its physiological consequences do not occur until there is an increase in the concentration of the protein encoded by the gene, the hypothesis that exercise increases the ability of muscle to utilize glucose and fatty acids via increased NR4A3 gene expression may not be correct.

As described above, we demondtrated that β-adrenergic stimulation up-regulates not only NR4A3 mRNA but also NR4A3 protein in the fast-twitch glycolytic triceps muscle (Fig. 2). Because exercise causes activation of the sympathetic nervous system (SNS) and promotes secretion of catecholamine such as adrenaline and noradrenaline [19], it would be supposed that exercise-activated SNS increases the expression of NR4A3 protein in the triceps muscle. However, in this study exercise increased NR4A3 mRNA but not protein in this muscle. Currently, we do not know the reason for this discrepancy; however, it might be possible that exercise-promoted secretion of catecholomine is not sufficient to increase NR4A3 protein, although we did not measure circulating catecholamine level in the exercised rats.

Chronically activating AMPK is suggested to be involved in exercise-induced chronic local adaptations in skeletal muscle, such as increases in GLUT-4, PGC-1α, and mitochondrial biogenesis, through the increase in AMP and decrease in phosphocreatine and ATP that occur in contracting muscles [20–23]. Furthermore, our previous study showed that pharmacological activation of AMPK increases NR4A3 mRNA expression in isolated skeletal muscle preparation [11]. These results suggest the possibility that chronically activating AMPK induces metabolic adaptations, e.g. increased PGC-1α, in skeletal muscles via increased NR4A3 gene expression. However, in this study, 7 days subcutaneous injection of AICAR, a pharmacological AMPK activator, did not increase NR4A3 protein expression in the soleus and triceps muscles, although chronic AICAR injection increased PGC-1α protein expression in these muscles (Tables 3 and 4). It is therefore unlikely that chronically activating AMPK induces metabolic adaptations in skeletal muscles via increased NR4A3 gene expression.

We found that in resting sedentary rats NR4A3 mRNA and protein expression were higher in the soleus muscle, which consists of >80% type I fibers [24] than in the triceps muscle, which consists of 70% type IIb fibers [25] (Fig. 5). Type I muscle fibers are heavily recruited (~7 h/day) in rats housed in standard laboratory cages, whereas type IIb fibers are rarely recruited (~2 min/day) [26]. Therefore, we hypothesize that the difference in the gene expression level of NR4A3 between different kinds of muscles can be attributed to the daily postural contractile activity level. This hypothesis is supported by our finding in this study that the loss of the postural support, i.e.

inactivity, because of hindlimb immobilization reduces NR4A3 mRNA and protein expression in the soleus muscle (Fig. 6). Because exercise does not result in increased NR4A3 protein expression in skeletal muscles, it is possible that reduced NR4A3 protein expression is involved in some specific cellular and molecular processes, explaining the responses during inactivity; i.e., inactivity is not the same as lack of exercise.

Previous studies have demonstrated that gene expression of NR4A3 is suppressed in skeletal muscles from rodent models of insulin resistance or diabetes [10]. The studies also showed that overexpression of NR4A3 markedly augmented insulin-stimulated glucose transport and GLUT4 translocation in adipocytes [10]. In our study, not only the NR4A3 protein level but also insulin-stimulated glucose transport was reduced in the soleus muscle of immobilized hindlimbs (see "Results"). These results suggest that inactivity-induced insulin resistance of glucose transport in postural muscle may be mediated by decreased NR4A3 protein expression level, at least in part.

In summary, this study demonstrated that, in rats, a single subcutaneous injection of clenbuterol, a β2-AR agonist, increased NR4A3 protein expression in the fast-twitch glycolytic triceps muscle. In contrast, acute and chronic exercise did not increase NR4A3 protein level in exercised muscle. Furthermore, chronic injection of AICAR, a pharmacological AMPK activator, did not increase NR4A3 protein in the soleus and triceps muscles. Finally, our study showed a high level of NR4A3 protein expression in the slow-twitch oxidative soleus muscle recruited during normal postural activity, and that loss of postural contractile activity because of hindlimb immobilization reduced the NR4A3 protein level in the soleus muscle. These findings provide evidence that, although exercise may not up-regulate NR4A3 protein expression, local postural contractile activity plays a crucial role in maintaining a level of NR4A3 protein expression in postural muscle.

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