Summary. Fifteen-week-old rats were subjected to unloading induced by hindlimb suspension for 3 weeks. The peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and forkhead box-containing protein O1 (FOXO1) mRNA levels and fiber profiles of the soleus and plantaris muscles in rats subjected to unloading (unloaded group) were determined and compared with those of age-matched control rats (control group). The body weight and both the soleus and plantaris muscle weights were lower in the unloaded group than in the control group. The PGC-1α mRNA was downregulated in the soleus, but not in the plantaris muscle of the unloaded group. The FOXO1 mRNA was upregulated in both the soleus and plantaris muscles of the unloaded group. The oxidative enzyme activity was reduced in the soleus, but not in the plantaris muscle of the unloaded group. The percentage of type I fibers was decreased and the percentages of type IIA and IIC fibers were increased in the soleus muscle of the unloaded group, whereas there was no change in fiber type distribution in the plantaris muscle of the unloaded group. Atrophy of all types of fibers was observed in both the soleus and plantaris muscles of the unloaded group. We conclude that decreased oxidative capacity and fiber atrophy in unloaded skeletal muscles are associated with decreased PGC-1α and increased FOXO1 mRNA levels.

Key words: FOXO1, Oxidative capacity, PGC-1α, Skeletal muscle, Unloading

Introduction

Skeletal muscle fibers are classified according to differences in adenosine triphosphatase (ATPase) activity after preincubation at different pH values. Type I (slow-twitch) and II (fast-twitch) fibers become ATPase negative and ATPase positive at pH 10.4, respectively (Hori et al., 1998). Type II fibers are subclassified into type IIA and IIB, according to ATPase stability at acidic pH levels; type IIA fibers become ATPase negative at a faster rate compared with type IIB fibers with increasing acidity of the preincubation medium. Type IIC fibers have also been identified, particularly in the antigravity soleus muscle of rats; these fibers are ATPase positive, regardless of the pH of the preincubation medium. The activities of oxidative enzymes in different types of fibers in the skeletal, perineal and heart muscles are well defined; oxidative enzyme activity is higher in type I, IIA and IIC fibers compared with type IIB fibers (Hiroyuki et al., 2000; Nakatani et al., 2003). However, oxidative enzyme activity is higher in type IIA and IIC fibers compared with type I fibers in the rat soleus muscle (Rivero et al., 1998, 1999). Fiber type distribution correlates with the contraction time and fatigue resistance of individual skeletal muscles.

Hindlimb unloading causes atrophy of all types of fibers in the rat soleus muscle (Ishihara et al., 1997, 2004). Transformation of fibers from type I to IIA and decreased oxidative enzyme activity in all types of fibers are also observed in the rat soleus muscle after hindlimb unloading. However, data pertaining to the levels of mRNAs related to morphology and metabolism in unloaded skeletal muscles are limited. Oxidative metabolism in skeletal muscles is largely regulated by the peroxisome proliferator-activated receptor γ (PPARγ)
coactivator-1α (PGC-1α) (Puigserver, 2005; Wende et al., 2005). High PGC-1α mRNA levels increase the percentage of high-oxidative type I and IIA fibers in skeletal muscles (Lin et al., 2002; Wu et al., 2002; Schuler et al., 2006). This finding suggests that PGC-1α regulates the proportion of high-oxidative fibers in skeletal muscles. Therefore, hindlimb unloading-induced transformation from high- to low-oxidative type and reduction of oxidative enzyme activity in individual fibers may be associated with decreased PGC-1α mRNA levels.

Forkhead box-containing protein O1 (FOXO1) inversely regulates the mass of skeletal muscles and gene expression in high-oxidative fibers (Kamei et al., 2004). The size of fibers in the rat soleus muscles overexpressing FOXO1 is small, regardless of fiber type (Kamei et al., 2004). Furthermore, the percentage of high-oxidative type I fibers in these muscles was low. Therefore, unloading-induced atrophy, transformation from high- to low-oxidative type and decreased oxidative enzyme activity in individual fibers may be associated with increased FOXO1 mRNA levels.

In the present study, PGC-1α and FOXO1 mRNA levels and fiber profile parameters, e.g., type distribution, cross-sectional area and oxidative enzyme activity, in the slow-twitch soleus and fast-twitch plantaris muscles of rats subjected to hindlimb unloading were determined and compared with those of age-matched control rats.

Materials and methods

All experimental and animal care procedures were conducted in accordance with the guidelines stated in the Guide for the Care and Use of Laboratory Animals issued by the Institutional Animal Experiment Committee of Kyoto University, Japan.

Experimental animals and treatments

Fifteen-week-old female F344/Jcl rats were used in the present study. The rats were divided into the control (n=6) and unloaded (n=6) groups. The unloading model of hindlimb suspension used in the present study was a modification of that described previously (Ishihara et al., 1997; Nagatomo et al., 2009b). This model allows free movement of the rat forelimbs and prevents resting of the hindlimbs on the floor of the cage. Hindlimb unloading was initiated when the rats were 15 weeks of age and was maintained for 3 weeks thereafter. Food and water were provided ad libitum to both groups. The room was maintained under a controlled 12 h light/dark cycle (dark period from 20h00 to 08h00) at 22±2°C with 45-55% relative humidity.

Analyses of PGC-1α and FOXO1 mRNA levels in the muscle

The soleus and plantaris muscles of both legs were removed under anesthesia with sodium pentobarbital (intraperitoneal administration, 50 mg/kg of body weight). Subsequently, excess fat and connective tissues were removed from the muscle and the wet weight of the muscle was measured. Total RNA was extracted from the left soleus and plantaris muscles using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Muscles were then treated with deoxyribonuclease I (Invitrogen). The first strand of cDNA was synthesized from 1.0 μg of total RNA using the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan). Gene expression was analyzed using real-time polymerase chain reaction (RT–PCR) performed on the LightCycler system DX400 (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq II (Takara Bio Inc.). The following primer sets were used:

PGC-1α forward, 5'-CGATGACCCTCCTCACACCA-3'; PGC-1α reverse, 5'-TTGGCTTGAGCATGTTGCG-3'; FOXO1 forward, 5'-AAGAGGCTCACCCTGTGCGC-3'; and FOXO1 reverse, 5'-GCATCCACCAAGAATTTC-3'. The mRNA levels of PGC-1α and FOXO1 were normalized to those of hypoxanthine phosphoribosyltransferase.

Biochemical analyses of the muscle

The right soleus and plantaris muscles were divided into two parts (proximal and distal) for biochemical and histochemical analyses. The distal part of the muscle was immediately frozen and homogenized in five volumes of ice-cold 0.3 M phosphate buffer (pH 7.4) using a glass tissue homogenizer. The final concentrations of the components in the reaction mixture were as follows: sodium succinate, 17 mM; sodium cyanide, 1 mM; aluminum chloride, 0.4 mM; and calcium chloride, 0.4 mM. The reduction of cytochrome c in this reaction mixture was analyzed using a spectrophotometer via observation of the increase in extinction at 550 nm. Succinate dehydrogenase (SDH) activity was calculated from ferricytochrome c concentrations and protein content.

Histochemical analyses of the muscle

The proximal part of the muscle was rapidly frozen in isopentane, which had been cooled previously using a mixture of dry ice and acetone, and stored at -80°C until further analyses. The muscle was mounted onto a specimen chuck using Tissue-Tek O.C.T. compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan). Serial transverse sections with a thickness of 10 μm were cut on a cryostat at -20°C. The sections were brought to room temperature, air dried for 30 min and preincubated in acidic (pH 4.5) and alkaline (pH 10.4) conditions for the subsequent assessment of ATPase activity (used for classification of fiber type). In each section, soleus muscle fibers were classified as type I (positive response to preincubation in pH 4.5 and negative response to preincubation in pH 10.4), type IIA (negative response to
preincubation at pH 4.5 and positive response to preincubation at pH 10.4) and type IIC (positive response to preincubation at pH 4.5 and pH 10.4) (Nakatani et al., 1999). Plantaris muscle fibers were classified as type I (positive response to preincubation at pH 4.5), type IIA (negative response to preincubation at pH 4.5) and type IIB (intermediate response to preincubation at pH 4.5) (Nakatani et al., 2000). A single common area was selected in each section and digitized as gray-level images using a computer-assisted image-processing system (Neuroimaging System, Kyoto, Japan). The cross-sectional area of the fibers was measured by tracing the outline of each fiber in the section. Fiber type distribution and cross-sectional area were determined in 500 fibers located in the central region of the muscle section.

The sections were stained for 10 min to determine fiber SDH intensity (Nakatani et al., 1999, 2000). SDH intensity was determined in the 500 above-mentioned fibers using a computer-assisted image-processing system (Neuroimaging System). Sectional images were digitized as gray-scale images. Each pixel was quantified as 1 of 256 gray levels; a gray level of 0 was equivalent to 100% light transmission, whereas a gray level of 255 was equivalent to 0% light transmission. The mean optical density (OD) of all pixels (which were converted to gray level values) within a fiber was determined using a calibration photographic tablet with 21 steps of gradient-density ranges and the corresponding diffused density values.

**Skeletal muscle property after unloading**

Fig. 1. Body weights (A) and soleus (B) and plantaris (C) muscle weights of the control and unloaded groups. Data are presented as the mean and standard deviation of six animals. Con, control; UnL, unloaded. *P<0.05 compared with the Con group.

Fig. 2. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α; A, C) and forkhead box-containing protein O1 (FOXO1; B, D) mRNA levels in the soleus (A, B) and plantaris (C, D) muscles of the control and unloaded groups. Data are presented as the mean and standard deviation of six animals. Con, control; UnL, unloaded. *P<0.05 compared with the Con group.
**Statistical analyses**

Means and standard deviations were calculated from the individual values using standard procedures. Student’s *t* test was used to evaluate the differences between the control and unloaded groups. A probability level of 0.05 was accepted as significant.

**Results**

**Body and muscle weights**

The body weight of the unloaded group was lower than that of the control group (Fig. 1A). Similarly, the soleus (Fig. 1B) and plantaris (Fig. 1C) muscle weights of the unloaded group were lower than those of the control group.

**PGC-1α and FOXO1 mRNA levels in the muscle**

The PGC-1α mRNA level in the soleus muscle of the unloaded group was lower than that of the control group (Fig. 2A). In contrast, the FOXO1 mRNA level in the soleus muscle of the unloaded group was higher than that of the control group (Fig. 2B).

There was no difference in PGC-1α mRNA level in the plantaris muscle between the control and unloaded groups (Fig. 2C). The FOXO1 mRNA level in the

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**Fig. 3.** Succinate dehydrogenase (SDH) activities in the soleus (A) and plantaris (B) muscles of the control and unloaded groups. Data are presented as the mean and standard deviation of six animals. Con, control; UnL, unloaded. *P*<0.05 compared with the Con group.

**Fig. 4.** Serial transverse sections of the soleus muscles of rats in the control (A–C) and unloaded (D–F) groups. Sections were stained for adenosine triphosphatase activity after preincubation at pH 10.4 (A, D) and pH 4.5 (B, E), as well as for succinate dehydrogenase activity (C, F). 1, type I; 2, type IIA; 3, type IIC. Scale bar: 100 µm.
plantaris muscle of the unloaded group was higher than that of the control group (Fig. 2D).

**SDH activity in the muscle**

The SDH activity in the soleus muscle of the unloaded group was lower than that of the control group (Fig. 3A). There was no difference in SDH activity in the plantaris muscle between the control and unloaded groups (Fig. 3B).

**Muscle fiber profiles**

The soleus muscles of the control and unloaded groups consisted of three types of fibers: types I, IIA and IIC (Fig. 4). The percentage of type I fibers in the soleus muscle of the unloaded group was lower than that of the control group (Fig. 5A). In contrast, the percentages of type IIA and IIC fibers in the soleus muscle of the unloaded group were higher than those of the control group. The cross-sectional areas of all types of fibers in the soleus muscle of the unloaded group were smaller than those of the control group (Fig. 5B). The SDH intensities of type IIA and IIC fibers in the soleus muscle of the unloaded group were lower than those of the control group (Fig. 5C).

The plantaris muscles of the control and unloaded groups consisted of three types of fibers: types I, IIA and IIB (Fig. 6). There was no difference in fiber type distribution in the plantaris muscle between the control and unloaded groups (Fig. 5D). The cross-sectional areas of all types of fibers in the plantaris muscle of the unloaded group were smaller than those of the control group (Fig. 5E). There was no difference in SDH intensity of all types of fibers in the plantaris muscle between the control and unloaded groups (Fig. 5F).

**Discussion**

**Fiber profiles in the muscle after hindlimb unloading**

In general, chronic hindlimb unloading results in decreased oxidative enzyme activity, transformation of fibers from type I to II and atrophy of all types of fibers in skeletal muscles, especially in the antigravity soleus muscle (Ishihara et al., 1997, 2004). The results of the present study were similar to those of previous studies (Ishihara et al., 1997, 2004), which used 2 weeks of hindlimb unloading: reduced oxidative enzyme activity (Fig. 3A), decreased percentage of type I fibers (Fig. 5A), increased percentage of type IIA and IIC fibers (Fig. 5A) and atrophy of all types of fibers (Fig. 5B).
were observed in the soleus muscle of rats after 3 weeks of hindlimb unloading.

Although the intramuscular activities in the soleus muscle of rats, which were recorded using electromyography, decreased in response to acute hindlimb unloading, the level of these activities recovered gradually during hindlimb unloading and reached the preloading level (Ohira et al., 2002). Therefore, we believe that the transformation of fibers from type I to II and atrophy of all types of fibers induced by hindlimb unloading are not caused by a decrease in neuromuscular activity, but are attributable to non-weight bearing.

There were no changes in oxidative enzyme activity (Fig. 3B), fiber type distribution (Fig. 5D) or fiber SDH intensity (Fig. 5F) in the plantaris muscle after hindlimb unloading. Furthermore, changes in weight and fiber cross-sectional area of the plantaris muscle in the unloaded group were small compared with those observed for the soleus muscle in the same group; the weight of unloaded soleus muscles was 55.8% that of control muscles (Fig. 1B), whereas the weight of unloaded plantaris muscles was 86.4% that of control muscles (Fig. 1C). Similarly, the area of type I, IIA and IIC fibers in unloaded soleus muscles was 33.8, 46.9 and 46.7% that of control muscles, respectively (Fig. 5B), whereas the area of type I, IIA and IIB fibers in unloaded plantaris muscles was 86.4, 86.8 and 85.7% that of control muscles, respectively (Fig. 5E). Fast-twitch muscles, which presumably contain type IIB fibers, exhibit relatively high-intensity and short-duration activity, which is required for actions demanding strength and power. Conversely, slow-twitch muscles, which presumably contain type I and IIA fibers, are capable of relatively low-intensity activity

**Fig. 6.** Serial transverse sections of the plantaris muscles of rats in the control (A, B) and unloaded (C, D) groups. The sections were stained for adenosine triphosphatase activity after preincubation at pH 4.5 (A, C) and for succinate dehydrogenase activity (B, D). 1, type I; 2, type IIA; 3, type IIB. Scale bar: 100 µm.
performed over a long period, such as walking and maintaining posture. This means that the function and metabolism of slow-twitch muscles are maintained under continuous loading onto the muscles. Therefore, it is suggested that hindlimb unloading had a greater effect on the slow-twitch soleus muscle compared with the fast-twitch plantaris muscle.

**PGC-1α mRNA level in the muscle after hindlimb unloading**

Upregulation of PGC-1α under the influence of a muscle creatine kinase promoter resulted in transformation of fibers from low- to high-oxidative type in transgenic mice (Lin et al., 2002). Enhanced PGC-1α mRNA levels cause increased mitochondrial content and results in the development of fibers with a highly oxidative phenotype (Wu et al., 2002; Schuler et al., 2006). Patients with type 2 diabetes exhibit a low PGC-1α mRNA level (Patti et al., 2003) and a decreased percentage of high-oxidative fibers (Márín et al., 1994; Hickey et al., 1995; Nyholm et al., 1997; Gaster et al., 2001) in their skeletal muscles compared with healthy individuals. These results suggest that PGC-1α regulates the proportion of high-oxidative fibers in skeletal muscles.

Our previous study (Adachi et al., 2007) showed that PGC-1α mRNA levels of low-oxidative fibers in the soleus muscle of diabetic Zucker rats were lower than those observed in the same type of fibers in the soleus muscle of non-diabetic rats. Furthermore, our recent studies (Nagatomo et al., 2009a, 2011) showed that PGC-1α mRNA levels in the soleus and plantaris muscles of diabetic Goto–Kakizaki (GK) rats were lower than those observed in non-diabetic rats. The soleus and plantaris muscles of GK rats presumably comprise low-oxidative fibers, whereas those of non-diabetic rats comprise both low- and high-oxidative fibers. Therefore, lower PGC-1α mRNA levels in skeletal muscles of diabetic rats seem to be associated with their fiber profiles, i.e., in this case, the low-oxidative fibers. The fiber type distribution present in skeletal muscles of diabetic rats is associated with blood glucose levels, but not with insulin levels, because the growth-related increase in blood glucose level is inversely correlated with the percentage of high-oxidative fibers in skeletal muscles (Yasuda et al., 2001, 2002).

An increase in PGC-1α mRNA level in skeletal muscles of rats with type 2 diabetes can result in an increased percentage of high-oxidative fibers, combined with improved insulin resistance and glucose tolerance. Exposure to hyperbaric oxygen reduces the growth-associated increase in blood glucose level of rats with type 2 diabetes (Yasuda et al., 2006, 2007). In addition, upregulation of the PGC-1α mRNA was observed in the soleus muscle of diabetic rats exposed to hyperbaric oxygen (Gu et al., 2010). The slow-twitch soleus muscle of diabetic rats exposed to hyperbaric oxygen comprises both low- and high-oxidative fibers, whereas that of diabetic rats not exposed to hyperbaric oxygen comprises only low-oxidative fibers. Similarly, an increased percentage of high-oxidative fibers and a decreased percentage of low-oxidative fibers combined with upregulation of the PGC-1α mRNA were observed in the fast plantaris muscle of diabetic rats after exposure to hyperbaric oxygen (Gu et al., 2010); these findings indicate that transformation of fibers from high- to low-oxidative type in both the slow- and fast-twitch muscles is inhibited by exposure to hyperbaric oxygen and is associated with the increase in PGC-1α mRNA level. Therefore, stimulation of pathways involving PGC-1α may increase the percentage of high-oxidative fibers and improves oxidative capacity in the skeletal muscles of diabetic rats.

In the present study, the PGC-1α mRNA was downregulated in the soleus muscle after hindlimb unloading (Fig. 2A). The oxidative enzyme activity in the soleus muscle (Fig. 3A) and the SDH intensity of type IIA and IIC fibers (Fig. 5C) decreased after hindlimb unloading. Furthermore, the percentage of type I fibers decreased and the percentages of type IIA and IIC fibers increased in the soleus muscle of rats in the unloading group (Fig. 5A). Conversely, there were no changes in PGC-1α mRNA level (Fig. 2C), oxidative enzyme activity (Fig. 3B), fiber type distribution (Fig. 5D) or fiber SDH intensity (Fig. 5F) in the plantaris muscle after hindlimb unloading. Based on these results, we conclude that the decreased oxidative capacity observed in unloaded skeletal muscles is associated with the decrease in PGC-1α mRNA level.

**FOXO1 mRNA level in the muscle after hindlimb unloading**

FOXO1 is a member of the forkhead-type transcription factors; the gene expression levels of these factors are upregulated in the liver, skeletal muscle and adipose tissue. FOXO1 appears to be a key molecule in the regulation of energy metabolism in skeletal muscles (Kamei et al., 2004). An increase in FOXO1 level of skeletal muscles results in a transition from carbohydrate to lipid oxidation via an increase in the expression of genes such as the pyruvate dehydrogenase kinase 4 (PDK4) gene, indicating that FOXO1 stimulates fatty-acid uptake and oxidation in skeletal muscle through the upregulation of PDK4 (Furuyama et al., 2003; Bastie et al., 2005; Chiba et al., 2009).

Overexpression of FOXO1 in response to starvation and caloric restriction promotes catabolism and impairs anabolism in skeletal muscle, and finally induces protein degradation and leads to the subsequent development of severe muscular atrophy (Kamei et al., 2004; Sandri et al., 2004; Stitt et al., 2004). This finding suggests that FOXO1 inversely regulates skeletal muscle mass and impairs skeletal muscle function. Mouse skeletal muscles overexpressing FOXO1 comprise a few type I fibers (Kamei et al., 2004), indicating that FOXO1 induces a decrease in percentage of high-oxidative fibers.
in skeletal muscles.

A previous study (Sandri et al., 2006) observed that a decrease in the levels of PGC-1α enhances the FOXO-dependent decrease in muscle mass. PGC-1α, which coactivates PPARα/δ in skeletal muscles, is regulated by FOXO1 (Wang et al., 2003; Southgate et al., 2005; de Lange et al., 2007). FOXO1 may inhibit the function of PGC-1α by binding to PGC-1α. In the present study, we expected decreased PGC-1α and increased FOXO1 mRNA levels in atrophied soleus and plantaris muscles, as changes in the levels of these two mRNAs are tightly linked. However, there was no change in PGC-1α mRNA level and oxidative, which FOXO1 mRNA. Therefore, we conclude of fibres in the soleus muscle of Zucker by binding to PGC-1α. In the present study, we enhances the FOXO-

Conclusion

We conclude that decreased oxidative capacity and fiber atrophy in unloaded skeletal muscles are associated with decreased PGC-1α and increased FOXO1 mRNA levels.

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