We examined the fiber profiles and the mRNA levels of peroxisome proliferator-activated receptors (PPAR\(\alpha\) and PPAR\(\delta/\beta\)) and of the PPAR\(\gamma\) coactivator-1\(\alpha\) (PGC-1\(\alpha\)) in the plantaris muscles of 15-week-old control (WR), metabolic syndrome (CP), hypertensive (SHR), and type 2 diabetic (GK) rats. The deep regions in the muscles of SHR and GK rats exhibited lower percentages of high-oxidative type I and IIA fibers and higher percentages of low-oxidative type IIB fibers compared with WR and CP rats. The surface regions in the muscles of CP, SHR, and GK rats exhibited lower percentages of high-oxidative type IIA fibers and higher percentages of low-oxidative type IIB fibers compared with WR rats. The muscles of SHR and GK rats had lower oxidative enzyme activity compared with WR rats. The muscles of SHR rats had the lowest PPAR\(\delta/\beta\) mRNA level. In addition, the muscles of SHR and GK rats had lower PGC-1\(\alpha\) mRNA level compared with WR and CP rats. We concluded that the plantaris muscles of rats with hypertension and type 2 diabetes have lower oxidative capacity, which is associated with the decreased level of PGC-1\(\alpha\) mRNA.

**Key words:** hypertension, oxidative capacity, PGC-1\(\alpha\) mRNA, plantaris muscle, type 2 diabetes

I. Introduction

Mammalian skeletal muscle fibers are classified according to differences in adenosine triphosphatase (ATPase) activity after preincubation at different pH values [7]. Type I (slow-twitch) and II (fast-twitch) fibers become ATPase negative and ATPase positive at pH 10.4, respectively. Type II fibers are subclassified as type IIA and IIB based on ATPase stability at acidic pH; type IIA fibers lose ATPase activity more quickly than type IIB fibers when the acidity of the preincubation medium increases. The activity of oxidative enzymes, e.g., succinate dehydrogenase (SDH), is well defined in different fiber types in skeletal, perineal, and heart muscles; oxidative enzyme activity is higher in type I and IIA fibers compared with type IIB fibers [8, 21]. The distribution of fiber types correlates with the contraction time and fatigue resistance of individual skeletal muscles. Skeletal muscles that presumably contain type I and IIA fibers are capable of relatively low-intensity activity performed over a long period, such as walking and maintaining posture. Conversely, skeletal muscles that presumably contain type IIB fibers exhibit relatively high-intensity and short-duration activity, which is required for actions demanding strength and power.

A number of experimental animal models of metabolic syndrome and lifestyle-related diseases, such as hypertension and type 2 diabetes, have been developed. However,
limited data are available on the skeletal muscle characteristics of these animal models. Our previous studies [34, 35] showed that the skeletal muscles of rats with type 2 diabetes exhibited low oxidative capacity compared with those of nondiabetic rats; the slow soleus and fast plantaris muscles of the type 2 diabetic rats contained a low percentage of high-oxidative fibers. These findings suggest that the decreased oxidative capacity of the skeletal muscles of type 2 diabetic rats may be associated with insulin resistance and impaired glucose metabolism.

Oxidative metabolism in skeletal muscles is largely regulated by several factors, such as the peroxisome proliferator-activated receptors (PPARs) and PPARγ coactivator-1α (PGC-1α) [31]. Enhanced mRNA levels of these receptors and coactivator induce an increase in the proportion of high-oxidative fibers in skeletal muscles, suggesting that the mRNA levels of the PPARs and PGC-1α in skeletal muscles are associated with muscle fiber profile parameters, such as type distribution and oxidative enzyme activity [12, 29, 32].

Our previous study [18] showed that the slow soleus muscles of type 2 diabetic rats exhibited a low percentage of high-oxidative fibers and a decreased PGC-1α mRNA level compared with those of nondiabetic rats. In the present study, we hypothesized that similar results would be observed in the fast plantaris muscles and that a reduction in the mRNA levels of the PPARs and PGC-1α in the slow and fast muscles may induce impaired glucose and lipid metabolism and lead to the development and progression of metabolic syndrome and lifestyle-related diseases.

II. 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 the Kyoto University (Kyoto, Japan).

Experimental animals and treatments

We housed 14-week-old male control Wistar (WR), metabolic syndrome (SHR/Ndmc-r-cp (cp/cp), CP), spontaneously hypertensive (SHR), and type 2 diabetic (Goto-Kakizaki, GK) rats (n=5 in each group) in individual, uniformly sized standard cages for 1 week. The room was maintained under a controlled 12 hr light/dark cycle (dark period from 00h to 08h00) at 22±2°C with 45–55% relative humidity. Food and water were provided ad libitum, and the food intake of each rat over a 24 hr period was measured daily.

Histochemical analyses of the muscle

At 15 weeks of age, the plantaris muscles of both legs were removed under sodium pentobarbital anesthesia (intraperitoneal administration, 50 mg/kg of body weight). Excess fat and connective tissues were removed from the muscle, and the wet weight of the muscle was measured. The right plantaris muscle was transversely divided into two parts (proximal and distal) for histochemical and biochemical analyses. The proximal part of the muscle was pinned to a corkboard lengthwise, rapidly frozen in isopentane that had been cooled with 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 OCT 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 solutions (pH 4.5) for the subsequent assessment of ATPase activity (for classification of the fibers into different types) [19, 20]. For the determination of ATPase activity, the samples were subjected to the following treatment: (1) preincubation for 5 min at room temperature in 50 mM sodium acetate and 30 mM sodium barbitol in distilled water (adjusted to pH 4.5 with HCl); (2) washing in five changes of distilled water; (3) incubation for 45 min at 37°C in 2.8 mM ATP, 50 mM CaCl₂, and 75 mM NaCl in distilled water (adjusted to pH 9.4 with NaOH); (4) washing in five changes of distilled water; (5) immersion for 3 min in 1% CaCl₂; (6) washing in five changes of distilled water; (7) immersion for 3 min in 2% CoCl₂; (8) washing in five changes of distilled water; (9) immersion for 1 min in 1% (NH₄)₂S; (10) washing in five changes of distilled water; and (11) dehydration in graded ethanol solutions and dipping in xylene. Fiber cross-sectional areas were measured using a computer-assisted image processing system (Neuroimaging System Inc., Kyoto, Japan). The cross-sectional area of the fibers was measured by tracing the outline of each fiber in the section. The muscle fibers in each section were classified as type I when they exhibited a dark staining intensity, type IIA when they exhibited a light staining intensity, and type IIB when they exhibited an intermediate staining intensity. Fiber type distribution and cross-sectional area were determined for approximately 200 fibers located in the deep (close to the bone), and for approximately 200 fibers located in the surface (away from the bone), regions of the muscle.

The sections were stained for 10 min to determine SDH intensity. The SDH reaction was visualized by incubating the sections in 0.1 M phosphate buffer (pH 7.6) containing 0.9 mM NaH₂PO₄, 0.9 mM 1-methoxyphenazine methosulfate, 1.5 mM nitroblue tetrazolium, 5.6 mM EDTA-disodium salt, and 48 mM succinate disodium salt [19, 20]. The reaction was terminated by washing in five changes of distilled water, and the sections were dehydrated in graded ethanol solutions and dipping in xylene. To prepare histochemical controls, either the succinate disodium salt or the nitroblue tetrazolium compound was excluded from the incubation medium. The intensity of the SDH in the fibers was determined using a computer-assisted image processing system (Neuroimaging System). The sectional images were digitized as gray-scale images. Each pixel was quantified as one of 256 of a gray level, with a gray level of 0 being equivalent to 100% light transmission and a gray level of 255 being equivalent to 0% light transmission. The mean optical
density (OD) of all pixels within a fiber was determined using a calibration photographic tablet with 21 steps of gradient density ranges and the corresponding diffused-density values.

**Biochemical analyses of the muscle**

The distal part of the right soleus 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 compounds in the reaction mixture were as follows: sodium succinate, 17 mM; NaCN, 1 mM; AlCl$_3$, 0.4 mM; and CaCl$_2$, 0.4 mM. The reduction of cytochrome c in this reaction mixture was analyzed using a spectrophotometer by observing the increase in the extinction at 550 nm. SDH activity was calculated based on ferricytochrome c concentrations and protein content [22].

**Analyses of muscle mRNA levels**

Total RNA was extracted from the left soleus muscle using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The muscle was 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). We analyzed gene expression using real-time polymerase chain reaction (RT-PCR) on a LightCycler system DX400 (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq II (Takara Bio Inc.). The mRNA levels of PPAR$_\alpha$, PPAR$\delta/\beta$, and PGC-1$\alpha$ were normalized to those of hypoxanthine phosphoribosyltransferase (HPRT). The following primer sets were used.

PPAR$_\alpha$ forward, 5'-CACCCTCTCTCCAGCTTCCA-3';
PPAR$_\alpha$ reverse, 5'-GCCTTGTCCCCACATATTCG-3';
PPAR$\delta/\beta$ forward, 5'-GCCCTGGTCCACATATTCCG-3';
PPAR$\delta/\beta$ reverse, 5'-AAGGAGATCAGGCTGACGTGAACGTG-3';
PPAR$\delta/\beta$ reverse, 5'-TGAGGAGAGGCTGCTGAA GT-3';
Pgc-1$\alpha$ forward, 5'-CGATGCCCTCTCCACACCA-3';
Pgc-1$\alpha$ reverse, 5'-TTGGCTGGCATGCTTG-3';
HPRT forward, 5'-CTCATGGACTTATTGACAGGAC-3';
HPRT reverse, 5'-GCAGGTCAGCAAAGAACTTATAGCC-3'.

**Statistical analyses**

The means and standard deviations were calculated from the individual values using standard procedures. All measured values are presented as the mean and standard deviation. One-way analysis of variance (ANOVA) was used to evaluate differences among the groups. When ANOVA analyses revealed the presence of significant differences, further comparisons were performed using Scheffé's post hoc tests. A probability level of 0.05 was accepted as significant.

### III. Results

**Body weight and food intake**

The body weight of the CP group was the greatest among the four groups (Fig. 1A). The body weight of the SHR and GK groups was lower than that of the WR group. The food intake of the CP group was the greatest among the four groups (Fig. 1B).

**Plantaris muscle weight**

The muscle weight of the SHR group was the lowest among the four groups (Fig. 1C). The muscle weight of the CP and GK groups was lower than that of the WR group. The relative muscle weight per body weight of the CP and SHR groups was lower than that of the WR and GK groups (Fig. 1D). The relative muscle weight per body weight of the GK group was lower than that of the WR group.

**Fig. 1.** Body weight (A), food intake (B), plantaris muscle weight (C), and relative plantaris muscle weight per body weight (D) in the control (WR), metabolic syndrome (CP), hypertensive (SHR), and type 2 diabetic (GK) groups. Data are presented as mean and standard deviation (n=5). 
BW, body weight; $p<0.05$ compared with WR; $p<0.05$ compared with WR, SHR, and GK; $p=0.05$ compared with WR, CP, and GK; $p<0.05$ compared with WR and GK.
Plantaris muscle fiber profiles

All groups had three types of fibers (type I, IIA, and IIB) in the deep regions of the muscles and two types of fibers (type IIA and IIB) in the surface regions of the muscles (Fig. 2).

In the deep regions of the muscles, the SHR and GK groups had lower percentages of type I and IIA fibers and higher percentages of type IIB fibers compared with the WR and CP groups (Fig. 3A).

In the surface regions of the muscles, the CP, SHR, and GK groups had lower percentages of type IIA fibers and higher percentages of type IIB fibers compared with the WR group (Fig. 3B).

In the deep regions of the muscles, the type I fibers of the CP group had smaller cross-sectional areas than those of the WR group (Fig. 3C). Furthermore, the type I fibers of the SHR and GK groups had smaller cross-sectional areas than those of the WR and CP groups. The type IIA fibers of the CP, SHR, and GK groups had smaller cross-sectional areas than those of the WR group. The type IIB fibers of the CP and GK groups had smaller cross-sectional areas than those of the WR group. Furthermore, the type IIB fibers of the SHR group had the smallest cross-sectional area recorded among the four groups.

In the surface regions of the muscles, the type IIA fibers of the CP, SHR, and GK groups had smaller cross-sectional areas than those of the WR group (Fig. 3D). The type IIB fibers of the CP and GK groups had smaller cross-sectional areas than those of the WR group. Furthermore, the type IIB fibers of the SHR group had smaller cross-sectional areas than those of the WR and CP groups.

In the deep and surface regions of the muscles, the type IIA and IIB fibers of the SHR and GK groups had lower SDH intensities than those of the WR and CP groups (Fig. 3E, F).

Plantaris muscle SDH activity

The muscles of the SHR and GK groups had lower SDH activities than those of the WR group (Fig. 4).

mRNA levels in the plantaris muscle

There were no differences in PPARα mRNA level of the muscles from the four groups (Fig. 5A). The PPARδ/β mRNA level in the muscles of the SHR group was the lowest.
among the four groups (Fig. 5B). The PGC-1α mRNA level in the muscles of the SHR and GK groups was lower than that of the WR and CP groups (Fig. 5C).

IV. Discussion

Skeletal muscles of patients with type 2 diabetes have a decreased oxidative capacity compared with those of healthy individuals, as the skeletal muscles of the former contain a low percentage of high-oxidative fibers [3, 6, 14, 23]. These findings suggest that the decreased oxidative capacity of skeletal muscles in patients with type 2 diabetes is associated with insulin resistance and impaired glucose metabolism.

Mammalian skeletal muscles are classified into two types, slow and fast muscles, based on their metabolic and functional properties. The normal activity patterns differ between slow and fast muscles; slow muscles exhibit an activity of relatively low-intensity and long-duration, which is required for performing functions against gravity, such as walking and posture maintenance, whereas fast muscles exhibit an activity of relatively high-intensity and short-duration, which is required for functions demanding strength and power. Unlike slow muscles, which presumably contain high-oxidative fibers, fast muscles presumably contain low-
oxidative fibers that exhibit decreased lipid storage capacity [2], insulin binding [1, 11], insulin-stimulated glucose uptake [9], and glucose-transport protein content [4, 15]. It is unclear whether these differences between the slow and fast muscles of rats with metabolic syndrome and lifestyle-related diseases affect fiber profiles and the mRNA levels.

In the present study, we focused on the mRNA levels of PPARs and PGC-1α in the skeletal muscles, as PPARα and PPARδ/β directly regulate the expression of certain nuclear-encoded mitochondrial genes and are closely related to the regulation of oxidative metabolism via PGC-1α [10, 26]. PGC-1α has emerged as a major regulator of multiple cellular processes, including oxidative metabolism and fiber type specialization in skeletal muscles, and is considered to be predominantly involved in the regulation of mitochondrial content and function, which are closely related to SDH activity in the tricarboxylic acid cycle [12, 13, 16, 25, 33].

Our previous study [18] showed that the slow soleus muscles of type 2 diabetic GK rats had lower PGC-1α mRNA level than those of control WR rats. In addition, the soleus muscles of WR rats had type I, IIA, and IIC fibers, whereas those of GK rats had only type I fibers, which exhibit a lower oxidative enzyme activity compared with type IIA and IIC fibers [8, 27, 28]. Therefore, we concluded that the lower PGC-1α mRNA level observed in the slow soleus muscles of rats with type 2 diabetes was associated with the exclusive presence of low-oxidative fibers. In the present study, we investigated the fiber profiles and the mRNA levels of PPARα, PPARδ/β, and PGC-1α in the fast plantaris muscles of metabolic syndrome, hypertensive, and type 2 diabetic rats to examine whether the fast plantaris muscles yield results that are similar to those observed for the slow soleus muscles.

**mRNA levels of PPARs in the fast plantaris muscle**

PPARs are expressed at high levels in the liver, kidney, skeletal muscle, adipose tissue, and vascular wall, which are tissues that are important for insulin sensitivity and resistance, glucose metabolism, and the regulation of blood pressure. PPARδ/β is the main transcription factor involved in fatty-acid oxidation in skeletal muscles and is activated by enzymes associated with long-chain fatty-acid β-oxidation [24].

In our previous study [18], the PPARα/β mRNA level in the slow soleus muscles of CP, SHR, and GK was lower than that observed in control WR rats. This result suggests that experimental animal models of metabolic syndrome and lifestyle-related diseases exhibit low PPARα/β mRNA level in skeletal muscles, irrespective of the type of disease. In the present study, the PPARα/β mRNA level in the fast plantaris muscles of SHR rats, but not of CP and GK rats, was lower than that observed in the muscles of WR rats. These results suggest that the plantaris muscles of SHR rats have a decreased capacity for fatty-acid oxidation. This finding is consistent with the result of our previous study using the soleus muscles of SHR rats [18]. However, in the present study, the PPARα/β mRNA level in the plantaris muscles of CP and GK rats did not differ from that observed in the muscles of WR rats. We did not elucidate why there were no differences in PPARα/β mRNA level in the plantaris muscles of CP and GK rats and SHR rats; however, we speculate that there are muscle-type specific differences in PPARα/β mRNA level in rats with metabolic syndrome and lifestyle-related diseases.

**mRNA level of PGC-1α in the fast plantaris muscle**

PGC-1α, which was originally identified as a nuclear receptor coactivator, is expressed in several tissues, including brown adipose tissue, skeletal muscle, heart, kidney, liver, and brain, and activates mitochondrial biogenesis and function [12, 17]. PGC-1α is also believed to be a key molecule involved in fatty-acid oxidation, as it interacts with PPARα to promote the transcription of nuclear genes that encode mitochondrial fatty-acid-oxidation enzymes [30].

The oxidative metabolism in skeletal muscles is largely regulated by PGC-1α, which coactivates PPARy [31]. Therefore, a decrease in PGC-1α expression is associated with a reduced oxidative metabolism, which may induce insulin resistance and impaired glucose metabolism, whereas
an increase in PGC-1α expression can result in an improvement of insulin resistance and impaired glucose metabolism. An increase in PGC-1α mRNA level induces an increase in the proportion of high-oxidative fibers in the skeletal muscles of transgenic mice [12]; this suggests that PGC-1α regulates the proportion of high-oxidative fibers in skeletal muscles. These findings were confirmed in skeletal muscles of diabetic rats; exposure to hyperbaric oxygen induced an enhancement of the PGC-1α mRNA level, a shift of fiber type from low- to high-oxidative, and an increase in fiber oxidative enzyme activity in the soleus and plantaris muscles of rats with type 2 diabetes [5, 36]. In the present study, a decrease in PGC-1α mRNA level was observed in the plantaris muscles of SHR and GK rats (Fig. 5C), and the oxidative capacity of the plantaris muscles of these rats was reduced (Figs. 3E, F and 4).

It is interesting to note that fiber type distribution (Fig. 3A, B; with the one exception that type IIA fiber percentages were lower and type IIB fiber percentages were higher in the surface regions of the muscles in CP rats), fiber SDH intensity (Fig. 3E, F), and muscle SDH activity (Fig. 4) in the plantaris muscles of CP rats were similar to those observed for WR rats. Furthermore, there were no differences in PPARα, PPARδ/β, or PGC-1α mRNA level in the plantaris muscles of CP and WR rats (Fig. 5). Our previous study [18] showed that 15-week-old CP rats had greater body weight and food intake compared with age-matched WR, SHR, and GK rats. Furthermore, CP rats had the highest levels of triglycerides, total cholesterol, LDL cholesterol, free fatty acids, and insulin. Blood glucose levels and systolic blood pressure were lower in CP rats than they were in GK and SHR rats, respectively, although these parameters were higher in CP rats compared with WR rats; therefore, 15-week-old CP rats were prediabetic and prehypertensive. We concluded that rats with metabolic syndrome under prediabetic and prehypertensive conditions have normal fiber profiles and mRNA levels of PPARs and PGC-1α in skeletal muscles.

Our previous study [18] demonstrated that the slow soleus muscles of rats with type 2 diabetes exhibited a decrease in PGC-1α mRNA level compared with control WR rats. In addition, the soleus muscles of GK rats had low-oxidative fibers exclusively. In the present study, the fast plantaris muscles of rats with hypertension and type 2 diabetes had an increased percentage of low-oxidative fibers, which was associated with a decrease in PGC-1α mRNA level. Therefore, we concluded that rats with hypertension and type 2 diabetes had decreased PGC-1α mRNA level, irrespective of muscle type. It is suggested that the decreased oxidative capacity of the skeletal muscles, which may be associated with insulin resistance and impaired glucose metabolism, is because of decreased PGC-1α mRNA level in rats with hypertension and type 2 diabetes.

**Fiber cross-sectional area in the fast plantaris muscle**

The muscle weight of CP, SHR, and GK rats was lower than that of WR rats (Fig. 1C). This result correlated well with the finding of smaller cross-sectional areas in all types of fibers in the muscles of CP, SHR, and GK rats compared with corresponding types of fibers in WR rats (Fig. 3C, D). It is suggested that the decreased muscle weight and fiber cross-sectional area observed in SHR and GK rats are because of their lower body weight compared with those of WR rats (Fig. 1A). In contrast, the body weight of CP rats was the highest among the four groups (Fig. 1A). We believe that the increased body weight of CP rats was because of obesity, which is associated with various metabolic disorders and cardiovascular diseases, and that obesity in CP rats did not affect plantaris muscle weight and fiber cross-sectional area. The findings obtained for the fast plantaris muscles differed from the results of our previous studies, which showed that the slow soleus muscles of CP rats were smaller compared with those observed for WR, SHR, and GK rats because of atrophy of all types of fibers in the soleus muscles of CP rats [18]. We suggest that the slow soleus muscles of CP rats induce a more severe atrophy because this type of muscle is an antigravity and posture muscle that is persistently active; therefore, the size of its fibers is more dependent on muscle activity and loading levels.

**Conclusion**

The present study showed that the deep regions of the plantaris muscles of SHR and GK rats had lower percentages of high-oxidative type I and IIA fibers and higher percentages of low-oxidative type IIB fibers than those of WR rats. The plantaris muscles of SHR and GK rats had lower oxidative enzyme activity compared with those of WR rats. Furthermore, the plantaris muscles of SHR and GK rats had lower PGC-1α mRNA levels than those of WR rats. We concluded that the plantaris muscles of rats with hypertension and type 2 diabetes have lower oxidative capacity, which is associated with the decreased level of PGC-1α mRNA.

**V. Acknowledgments**

This study was supported by a grant from the Japan Aerospace Exploration Agency.

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