Skeletal Muscle Hypertrophy after Chronic Restriction of Venous Blood Flow in Rats

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ABSTRACT

KAWADA, S., and N. ISHII. Skeletal Muscle Hypertrophy after Chronic Restriction of Venous Blood Flow in Rats. Med. Sci. Sports Exerc., Vol. 37, No. 7, pp. 1144–1150, 2005. Purpose: Some previous studies have shown that resistance exercise training with venous occlusion causes an enhanced hypertrophy in human muscles. To investigate the effects of blood flow on muscular size at either cellular or subcellular level, we developed an animal model in which several veins from hindlimb muscles of the rat are surgically crush-occluded. Methods: Twenty-four male Wister rats were randomly assigned into either a group for sham operation (sham group) or a group for venous occlusion (experimental group; N = 12 for each group). Fourteen days after the operation, plantaris, soleus, gastrocnemius, extensor digitorum longus, and tibialis anterior muscles were dissected from hindlimbs and subjected to morphological and biochemical analyses. Results: Fourteen days after the operation, the muscles expect for soleus showed similar increases in wet weight/body weight (by 7–12%) as compared with the sham-operated group (P < 0.05). Further analyses on the plantaris muscle showed increases in muscle dry weight/ body weight (by 10%) and the concentrations of myofibrillar protein (by 23%), glycogen (by 93%) and lactate (by 23%) after the operation (P < 0.05). Mean fiber cross-sectional area was larger by 34% in the experimental group than in the sham-operated group (P < 0.01). The content of HSP-72 increased, whereas that of myostatin protein decreased (P < 0.01). The expression of nitric oxide synthase-1 (NOS-1) mRNA increased (P < 0.01), whereas that of IGF-1 mRNA showed no significant change (P = 0.36). Although the muscle nitric oxide (NO) concentration tended to increase, but the change was not significant (P = 0.10). Conclusions: Changes in muscle blood flow may affect the muscular size through actions of HSP-72, myostatin, and NOS-1. Key Words: VENOUS OCCLUSION, MYOSTATIN, HEAT SHOCK PROTEIN-72, NITRIC OXIDE SYNTHASE-1

Protein synthesis in skeletal muscle is stimulated by various factors including hormones, metabolic demand, and mechanical overload. Among these factors, strong mechanical stress, for instance, that associated with heavy resistance exercise, has been thought to play a primary role in inducing increases in size and strength of skeletal muscle. Previous studies have demonstrated that resistance exercise training with an intensity exceeding 65% of the maximum strength (one-repetition maximum; 1RM) is required for gaining muscular size and strength (15). Such a strenuous exercise would simultaneously activate secretions of anabolic hormones and anaerobic energy metabolism, though the precise roles played by these factors in muscular hypertrophy have not been fully understood. On the other hand, exercises with much lower intensity and larger volume result in an improvement of muscle oxidative capacity without considerable effect on muscular size (9).

Several recent studies have shown that a low-intensity resistance exercise combined with moderate vascular occlusion effectively causes increases in muscular size, strength, and glycogen content in humans (6,27). The acute effect of this exercise has been characterized by marked increases in serum concentrations of norepinephrine, growth hormone, and lactate (27). In addition, the electrical activity of muscle is considerably elevated during the exercise, suggesting that a large number of fast-twitch fibers are recruited even under the load as small as 20% 1RM. Such a resistance exercise with small mechanical stress is expected to be useful for rehabilitation and training for elders. Although some studies have shown that a similar training with occlusion causes metabolic and neuromuscular adaptations, its muscle-trophic effect has not been fully established (6,18). Thus, it would be worth investigating either cellular or subcellular events that may occur after the resistance exercise with occlusion.

In the resistance exercise with occlusion, suppression of muscular blood flow and resulting changes in environment...
around muscle fibers, for instance, hypoxia or accumulation of metabolic subproducts, may cause changes in up-stream regulators of protein synthesis. Among these regulators, heat shock protein-72 (HSP-72), myostatin, insulinlike growth factor-1 (IGF-1), and nitric oxide synthase-1 (NOS-1) are thought to be involved in exercise-induced muscular hypertrophy. HSP-72, one of the stress proteins, has been shown to play as a chaperone against ischemia in such cells as myoblast and myocardium (11,14). In humans, this protein has been shown to increase in muscles of patients with arterial occlusive disease (12). Myostatin, a member of the transforming growth factor-β (TGF-β) superfamily, is a potent negative regulator of muscle growth (16), and its content within muscle has been shown to decrease in response to mechanical stress (10). It has also been reported that the follistatin related gene protein, an antagonist to myostatin increases, whereas the activin IIb receptor, a receptor of myostatin decreases after heavy resistance training (30). On the other hand, IGF-1 is one of the critical growth mediators of muscle and other tissues. Overexpression of IGF-1 with viral mediated IGF-1 gene transfer has been shown to cause muscular hypertrophy (5). NOS-1 produces nitric oxide (NO) from L-arginine, which may activate ERK1/2, one of the mitogen-activated protein (MAP)-kinase family, thereby finally stimulate muscle growth through phosphorylation cascade reactions (28). If the suppression of muscular blood flow actually causes muscular hypertrophy, it may also cause changes in the intramuscular concentrations of some of these substances.

An appropriate animal model is of great advantage to study the subcellular mechanisms underlying the effects of blood flow on the size of muscle. Thus the present study aimed to develop a novel model with hindlimb muscles of the rat. To suppress moderately the muscular blood flow, some veins from hindlimb muscles were occluded by a surgical operation. Fourteen-day normal cage activity flow, some veins from hindlimb muscles were occluded by means of a heated metal needle under anesthesia with an intraperitoneal injection of sodium pentobarbital (100 mg · kg⁻¹ body weight). The occluded points were about 1 cm apart from the junction where the described veins join vena femoralis. In addition, vena femoralis was also occluded at the point about 1 cm apart from its junction to the region subinguinalis (Fig. 1). In sham-operated group, the skin of right hindlimb was cut open and then sutured. The contralateral muscles in the experimental group were not used as control, because they might be overloaded in compensation to occluded muscles.

After the operation, animals of both experimental and sham-operated groups were kept in rat cages for 2 wk. This period was based on our preliminary observation that blood vessels were regenerated rapidly around crush-occluded portions and blood circulation was almost recovered at >3 wk after the operation. Two weeks after the operation, the rats were sacrificed by an intraperitoneal injection of sodium pentobarbital, and hindlimb muscles [plantaris, soleus, gastrocnemius, extensor digitorum longus (EDL), and tibialis anterior (TA)] were dissected and weighed. For plantaris and soleus muscles, six samples from each group were dried at 50°C for 24 h to measure dry weight. The dry weight was the same when measured 16 h and 24 h after the initiation of treatment, indicating that the specimen was almost completely desiccated. A part of the remaining samples was snap frozen in liquid nitrogen for biochemical analysis, while the other part was frozen in cold isopentane for morphological observations. Both samples were stored at −80°C until analysis.

**Measurements of myofibrillar protein, glycogen, lactate, and nitric oxide concentrations.** For plantaris and soleus muscles, the amount of total protein remained after the preparation for Western blot analysis was determined spectrophotometrically using a protein determination kit (Bio-Rad, Richmond, CA). The total protein content was expressed relative to muscle wet weight. The muscle glycogen and lactate concentrations were measured as described by Russel and Taylor (23) and Gutmann and Wahl-

**MATERIALS AND METHODS**

**Animals.** Male Wister rats (age, 11 wk; body weight, 260–300 g; N = 24) were used. They were housed in an animal room with regulated temperature (22°C), humidity (60%), and illumination cycles (12-h light and 12-h dark). They were allowed to eat commercial rat chow (CLEA, Japan) and drink water ad libitum. Rat care and all experimental procedures employed were in accordance with the policy statement of the American College of Sports Medicine on research with experimental animals. The study was approved by the Ethical Committee for Animal Experiments at the University of Tokyo.

**Venous occlusion.** The rats were randomly assigned into a group for sham operation (sham group) or a group for venous occlusion (experimental group; N = 12 for each group). In the experimental group, vena saphena magna, vena saphena accessoria medialis, vena circumflexa ilium superficialis, and vena epigastrica superficialis in the right hindlimb were crush-occluded by means of a heated metal needle under anesthesia with an intraperitoneal injection of sodium pentobarbital (100 mg · kg⁻¹ body weight). The occluded points were about 1 cm apart from the junction where the described veins join vena femoralis. In addition, vena femoralis was also occluded at the point about 1 cm apart from its junction to the region subinguinalis (Fig. 1). In sham-operated group, the skin of right hindlimb was cut open and then sutured. The contralateral muscles in the experimental group were not used as control, because they might be overloaded in compensation to occluded muscles.

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efeld (8). For glycogen assay, 15 mg of muscle sample was dissolved in 30% KOH saturated with Na2SO4 at 100°C for 15 min and then cooled on ice. To precipitate glycogen, 95% ethanol (1.2 volumes) was added to the sample, and the sample was centrifuged at 2500 × g (20°C) for 20 min. The glycogen precipitate was dissolved in 400-μL distilled water; 100 μL of 5% phenol solution and 1 mL of H2SO4 were added into 200 μL of the obtained glycogen solution, which was then incubated at room temperature for 10 min. The glycogen concentration was determined spectrophotometrically at 490 nm. For lactate assay, another 15 mg of muscle sample was homogenized in 300 μL of 0.6 N HClO4 and centrifuged at 10,000 × g (20°C) for 5 min; 50 μL of the supernatant, 100 μL of 40 mM nicotinamide-adenine dinucleotide (Sigma-Aldrich Co.), and 3 μL of lactate dehydrogenase (10 mg · mL⁻¹; Roche Diagnostics) were added into 2 mL of a buffer (0.5 M glycine, 0.0127 M EDTA, and 0.4 M hydrazine, pH 9.0), and then incubated at room temperature for 30 min. The lactate concentration was determined spectrophotometrically at 340 nm. For NO assay, 30 mg of muscle sample was homogenized in 300 μL of 0.6 N HClO4 and centrifuged at 15,000 × g (20°C) for 15 min and then cooled on ice. To precipitate glycogen, 95% ethanol (20°C) for 20 min. The lactate concentration was determined spectrophotometrically at 340 nm. For NO assay, 30 mg of muscle sample was homogenized in 300 μL of 0.6 N HClO4 and centrifuged at 15,000 × g (20°C) for 15 min. The supernatant was filtrated with microcon YM-10 (Millipore Co.) at 5000 × g (4°C) for 60 min. Then NO concentration in the filtrated sample was measured using a NO2/NO3 Assay Kit-CII, FII (Dojindo, Japan) according to the manufacture’s procedure.

**Morphological observation.** Cross-sections (10-μm thick) were cut from frozen samples of plantaris muscles and stained with hematoxylin-eosin (Sakura Finetek Japan Co.) according to the standard procedure. Cross-sectional area of muscle fibers was determined using the NIH image (version 1.61; National Institutes of Health, Bethesda, MD).

**Western blot analysis.** Tissue samples were homogenized in a buffer containing 10-mM Tris-HCl (pH 7.4), 5% sodium dodecyl sulfate, and 5% 2-mercaptoethanol. The homogenate was boiled for 3 min, then centrifuged at 13,000 × g (4°C) for 15 min. The supernatant was removed and its protein concentration was determined using a protein determination kit (Bio-Rad, Richmond, CA). Then equal amounts of extracted muscle proteins (100 μg total protein) were separated by sodium dodecyl sulfate-polyacrylamide (12%) gel electrophoresis (SDS-PAGE). Proteins on a gel were transferred onto poly vinylidene difluoride (PVDF) membrane (ATTO, Japan) electrophoretically. The following primary antibodies were used for immunoblotting: HSP-72, 1:10,000 dilution of purified rabbit polyclonal anti-HSP70 antibody (StressGen Biotechnologies Co.); myostatin, 1:250 dilution of antimyostatin antibody as described previously (10). And then horseradish peroxidase (HRP)-conjugated goat antirabbit IgG [1:10000 (American Qualex, CA)] was reacted as a secondary antibody. After incubation with the secondary antibody, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Perbio Science Co., Rockford) according to the manufacture’s recommendation. Band densities were determined by using the NIH image, and used as indicators of HSP-72 and myostatin contents in the same amount of solubilized proteins, respectively. To obtain the same reaction condition, samples of a series of experiment were analyzed on the same gels and membranes.

**Measurements of NOS-1 and IGF-1 mRNA.** For reverse transcriptase-polymerase chain reaction (RT-PCR), RNA was extracted from plantaris muscles using total RNA extraction kit (ISOGEN, NIPPON GENE, Japan). Extracted total RNA was treated with RNase-free DNase-I (RQ-1; Promega, Madison, WI) at 37°C for 1 h to eliminate genomic DNA contamination. The quantity of extracted RNA was determined by absorbance at 260 nm. Complementary DNA (cDNA) was reverse transcribed (RT) from 5 μg of total RNA using 0.5-μg oligo (dT) and superscript II reverse transcriptase (Invitrogen, Netherlands) in a 20-μL volume. To confirm the RT reaction, PCR amplification was performed on the resulting RT product using primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as a positive control. And then PCR amplification for NOS-1 and IGF-1 were performed on the RT product using specific primers. Primers for NOS-1 and IGF-1 were synthesized on the basis of published information (19, 22). The sequences of the primers are listed in Table 1. The linear portion of the amplification curve for each transcript was defined and then used to determine the appropriate number of PCR amplification cycles in the RT-PCR analysis. PCR for NOS-1 started with an initial denaturation at 94°C for 5 min, followed by 37 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension cycle at 72°C for 7 min. PCR for IGF-1 started with an initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 1 min, and a final extension cycle at 72°C for 7 min. The amplified PCR products for each gene were visualized on 1.5% agarose gels stained with ethidium bromide. As described in western blot analysis, band density was determined by using the NIH image. The mRNA concentrations of both genes were normalized with respect to GAPDH.

**Statistical analysis.** Differences between groups were examined with Student’s t-test. P < 0.05 was regarded as statistically significant.

**RESULTS**

**Muscular size and concentrations of myofibrillar protein, glycogen and lactate.** Fourteen days after the operation, plantaris, gastrocnemius, EDL, and TA from the occluded limbs (right) showed significant increases (7–12%) in wet weight per body weight as compared with
those in sham-operated group. Conversely, the soleus muscle showed no significant increase. For muscle dry weight, the plantaris muscle showed an increase (10%), whereas the soleus muscle did not. In the plantaris muscle, concentrations of total protein, glycogen and lactate also increased by 23, 93, and 23% respectively, and the changes were significant as compared with the sham-operated group (Table 2).

**Muscle-fiber diameter.** Typical appearances of cross-sections of the plantaris muscle are shown in Figure 2. In venous-occluded muscle, the muscle fibers exhibited hypertrophy without any sign of abnormality, that is, edema, necrosis, and apoptosis (Figs. 2A and B). On the other hand, when a complete occlusion of both arteries and veins was made for only 30 min by strong ligation with a thread at the proximal end of hindlimb, the muscle exhibited an abnormal appearance and a number of necrotic fibers were observed on the next day (Fig. 2C). The distribution of fiber cross-sectional area in sham and experimental groups showed a shift towards the larger value in the experimental group (Fig. 3). The mean fiber cross-sectional area was larger by 34% in experimental group than in sham-operated group (4980 ± 2281 μm² vs 3704 ± 1326 μm²; N = 750; P < 0.01).

**HSP-72, myostatin, NOS-1, IGF-1, and NO.** In the plantaris muscle, venous occlusion caused a significant increase in the content of HSP-72 (Fig. 4A). On the other hand, the content of myostatin decreased significantly (Fig. 4B). RT-PCR analysis showed that, in venous-occluded muscle, NOS-1 mRNA expression was significantly up-regulated (Fig. 5A). Although muscle NO concentration tended to show an increase, the change was not significant (P = 0.10) due mainly to the large variation (Table 2). Also, IGF-1 mRNA expression showed an insignificant increase (P = 0.36) after venous occlusion (Fig. 5B).

**DISCUSSION**

The present study showed that chronic restriction of muscular venous blood flow induces muscle hypertrophy with

| Table 2. Effects of venous occlusion on body weight, muscle weight, concentrations of myofibrillar protein, muscle glycogen, lactate, and nitric oxide. |
|---------------------------------|-----------------|-----------------|
| **Sham Group**                  | **Experimental Group** |
| Body weight (g)                 |                   |
| Preexperiment                   | 279 (2.7)         | 281 (4.0)       |
| Postexperiment                  | 330 (4.7)         | 336 (3.4)       |
| Muscle wet weight/body weight (mg · g⁻¹) |                   |
| Plantaris                       | 0.983 (0.023)     | 1.103 (0.007)*  |
| Gastrocnemius                   | 4.708 (0.045)     | 5.075 (0.093)*  |
| EDL                             | 0.458 (0.005)     | 0.492 (0.011)*  |
| TA                              | 1.583 (0.015)     | 1.774 (0.037)*  |
| Soleus                          | 0.389 (0.010)     | 0.389 (0.007)   |
| Muscle dry weight/body weight (mg · g⁻¹) |                   |
| Plantaris                       | 0.254 (0.003)     | 0.279 (0.005)*  |
| Soleus                          | 0.100 (0.002)     | 0.100 (0.002)   |
| Myofibrillar protein concentration (mg · g⁻¹ wet tissue) |                   |
| Plantaris                       | 196 (17.8)        | 242 (9.0)*      |
| Muscle glycogen concentration (μmol · g⁻¹ wet tissue) |                   |
| Plantaris                       | 12.7 (1.3)        | 24.5 (3.6)*     |
| Muscle lactate concentration (μmol · g⁻¹ wet tissue) |                   |
| Plantaris                       | 32.1 (1.9)        | 39.4 (1.2)*     |
| Muscle NO concentration (nmol · g⁻¹ wet tissue) |                   |
| Plantaris                       | 29.5 (5.7)        | 48.7 (8.8)      |

Values of SEM are shown in parentheses. Asterisks indicate significant difference (P < 0.05) as compared with sham group. EDL, extensor digitorum longus; TA, tibialis anterior.
normal cage activity in the hindlimb muscles but not the soleus muscle (Table 2). This suggests that some major muscles of the hindlimb responded with hypertrophy to the operation in a similar manner, and there was not any unexpected change in muscle loading due to the treatment. In accordance with previous studies (4), myosin ATPase staining showed that the percentage of Type 1 fibers in the soleus muscle was much larger than in other hypertrophied muscles (data not shown). Therefore, the effects of venous occlusion may depend on the muscle-fiber composition.

The hypertrophied plantaris muscle showed increases in the concentrations of glycogen and lactate. Previous studies have shown that resistance training with vascular occlusion also causes muscle glycogen in humans (6). This effect may involve, at least partially, a hypoxia-induced activation of muscle glucose uptake through the translocation of the glucose transporter GLUT-4 (7). On the other hand, chronically elevated concentration of lactate is likely caused by either restricted clearance of lactate from the muscle or elevated activity of fast-twitch fibers. Therefore, the increases in muscle glycogen and lactate suggest that muscle blood flow was suppressed during the experimental period.

The hypertrophy of the plantaris muscle was associated with an increased content of HSP-72. It has been shown that exercise training also causes an increase in muscle HSP-72 content (20). Although the exact roles of HSP-72 in protein metabolism in skeletal muscle are not fully understood, it may stabilize both existing and newly synthesized proteins against several stressors associated with exercise training. It has been shown that in a variety of in vivo and in vitro experiments, HSP-72 is induced by such stressors as heat, ischemia, hypoxia, and free radicals, and acts as chaperone to prevent misfolding or aggregation of proteins (11,14). In addition, a short-time exposure of skeletal muscle to heat (41°C) has been shown to cause an increase in HSP-72 content and attenuate the atrophy when the muscle is subsequently subjected to unloading (21). Therefore, an increased production of HSP-72 may play a part in the present, occlusion-induced muscular hypertrophy.

An increased expression of NOS-1 within muscle fibers may also play an important role in muscular hypertrophy, because several recent studies have shown that NO stimulates the muscle growth (3,28). A NOS inhibitor N \( N^\text{G} \)-nitro-L-arginine methyl ester hydrochloride suppresses the activation of muscle satellite cells, whereas a NO donor, sodium nitroprusside dihydrate, promotes their proliferation (28). NO has also been shown to mediate the expression of vinculin and talin, which are cytoskeletal proteins responsible for force transmission in a variety of cells (29). In spite of the increased NOS-1 expression, muscle NO concentration did not show a significant increase due to large variations (Table 2). The life span of NO is so short that the present study evaluated its concentration indirectly by measuring its oxidation products. Therefore, obtained values might be resulted from production and breakdown of NO, both of which might be influenced by the restriction of muscle blood flow.

The hypertrophy of the plantaris muscle was associated with the decrease in muscle myostatin content. Myostatin gene has two binding sites for NF-kB in its regulatory regions (13), which has been shown to be activated by reactive oxygen species (ROS) produced in hypoxic condition (24). As has been well known, myostatin strongly inhibits the growth of muscle, and mutations of its gene result in overgrowth of musculature in mice, cattle and humans (16,17,25). In addition, muscle myostatin content has been shown to decrease in response to mechanical overloading, implying its role in regulating the muscle size in normal organisms (10).

A number of studies have so far shown that mechanical overloading upregulates IGF-1 and causes hypertrophy in skeletal muscle (1,2). It has also been shown that an increase
in muscle IGF-1 production induced by gene transfer with a viral injection method strongly enhances the growth of muscle (5). The IGF-1 induced muscular hypertrophy is associated with the increase in muscle-fiber diameter and the presence of central nuclei, suggesting the involvement of the proliferation of muscle-satellite cells and their fusion into parent muscle fibers (5). In the present study, the venous occlusion did not cause a significant increase in IGF-1 expression, and the central nuclei were not clearly seen (Figs. 2 and 5B). These results suggest that IGF-1 would not be always essential for muscle hypertrophy, if such factors as myostatin, HSP-72, and NOS-1 would change in favor of the muscular growth.

The present animal model may not be ideal for studying the mechanism underlying the resistance exercise with occlusion for humans, because both exercise and occlusion stimuli last for only a few minutes (5–10 min) in the latter (27). However, it may uncover some possible processes that are activated after the resistance exercise with occlusion. In addition, normal strenuous exercise training may share some of these processes, because during muscular activities, the muscle is subjected to repeated ischemia and reperfusion in phase with contraction and relaxation, respectively (26).

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