Local Changes of IGF-I mRNA, GH Receptor mRNA, and Fiber Size in Rat Plantaris Muscle Following Compensatory Overload

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Abstract: We investigated whether local insulin-like growth factor-I (IGF-I) mRNA and growth hormone (GH) receptor mRNA expressions in plantaris muscle were related to the region-specific hypertrophy following compensatory overload. Adult male normal or hypophysectomized rats were subjected to unilateral distal-half removals of the gastrocnemius and soleus muscles. The contralateral hindlimb was used as the control. Two weeks later, fiber areas in the distal and proximal parts of the plantaris muscle were measured. All the fiber areas measured in the distal part of the plantaris muscle in normal and hypophysectomized rats were significantly increased following the compensatory overload. In the proximal part, fiber areas of type I, IIA, and IIC were significantly increased, but fiber area of type IIB did not change. IGF-I mRNA expressions in the distal and proximal

parts were increased 3 d after the compensatory overload in normal and hypophysectomized rats. The increase of IGF-I mRNA expression in the distal part 3 d after compensatory overload was greater than those in the proximal part. IGF-I mRNA expressions in the distal and proximal parts were increased 14 d after the compensatory overload in hypophysectomized rats, but not in normal rats. GH receptor mRNA expressions were decreased following compensatory overload, and almost disappeared 14 d after the compensatory overload in hypophysectomized rats. Thus muscle fiber hypertrophy following compensatory overload was different among the parts in a muscle and IGF-I mRNA was expressed in concert with the region-specific hypertrophy, but not GH receptor mRNA. [*Japanese Journal of Physiology*, 53, 53–60, 2003]

Key words: IGF-I, GH receptor, hypertrophy, hypophysectomy, muscle fiber.

Skeletal muscle is hypertrophied by compensatory overload [1–3]. The magnitude of the hypertrophy after compensatory overload is different between the regions in a rodent muscle [4]. In humans, region-specific hypertrophy exists in consecutive cross-sectional

areas of the elbow flexors [5]. Region-specific hypertrophy in a muscle is related to the different muscle fiber recruitment pattern [6, 7]. However, what causes region-specific hypertrophy is unclear.

Many studies have shown that growth hormone

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(GH) and insulin-like growth factor-I (IGF-I) have an important role in the growth of skeletal muscle. Growth of skeletal muscle is severely retarded during development in hypophysectomized rats [3, 8] and administration of GH and IGF-I in hypophysectomized rats restores growth of skeletal muscle [8–10]. Generally, GH is thought to stimulate the growth of skeletal muscle by way of IGF-I mainly produced in the liver. Also, GH may contribute directly to the growth of skeletal muscle, as local injections of GH stimulate muscle growth [11] and the expression of GH receptor mRNA is increased in regenerating muscle [12]. IGF-I contributes to the growth of skeletal muscle due to GH-dependent endocrine action, as well as GH-independent autocrine/paracrine action [13–15]. Thus, there is a possibility of GH and IGF-I being associated with the region-specific hypertrophy, however, the relationships between GH or IGF-I and the region-specific hypertrophy have not been investigated.

Isgaard *et al.* [12] reported that GH receptor mRNA expression enhanced in volume overloaded cardiac muscle. Jennische and Andersson [16] showed that the expression of GH receptor mRNA increases in regenerating skeletal muscle after ischemic damage. These observations suggest that GH receptor may be related to muscle growth during various conditions, included compensatory overload. However, GH receptor mRNA expression during compensatory overload is not investigated, and whether muscle hypertrophy after compensatory overload is related to the expression of the GH receptor remains unclear.

Consequently, this study attempted to measure IGF-I mRNA and GH receptor mRNA expressions on the region-specific hypertrophy in plantaris muscle after compensatory overload.

MATERIALS AND METHODS

Animal care and surgical procedures. Male Wistar strain rats weighing ~170 g were used. Twelve rats were subjected to a hypophysectomy operation at 10 weeks of age (hypox rats). Twelve other rats were used as the control (normal rats). One week later, all rats suffered from compensatory overload of the plantaris muscle by unilateral removals of the gastrocnemius and soleus muscles (Hypox-OL and Normal-OL). The contralateral sham-operated muscles were used as the control (Hypox-Cont, Normal-Cont). All animals were housed in cages at constant room temperature ($22 \pm 2^\circ\text{C}$) and fed *ad libitum*. All experiments and procedures were performed according to the Guideline for the Care and Use of Laboratory Animals in Health Sciences University of Hokkaido.

The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital ($40 \text{ mg} \cdot \text{kg}^{-1}$ body mass). Hypophysectomy was carried out using the transauricular technique described by Koyama [17]. A puncture needle was inserted into the external auditory canal in the anesthetized animals, which pierced the thinner part of the bone wall of the tympanum. The inner cylinder of the injector fixed to the needle was pulled out slightly to create negative pressure and the pituitary body was then aspirated. The pituitary body was identified by the pinkish color of the anterior lobe. Success of the surgical operation was confirmed by direct observation showing the lack of pituitary gland at the end of this experiment.

Compensatory overload was performed by the unilateral distal-half removals of the gastrocnemius and soleus muscles using the modified method described by Ianuzzo and Chen [18]. The ablation operation was initiated with a longitudinal incision through the skin and fascia along the posterior aspect of the tibia. The lateral and medial heads of the gastrocnemius and soleus muscles were carefully isolated by way of blunt dissection and the distal halves of the muscles were removed. Vasculature and nerves associated with the plantaris muscles were carefully preserved.

The plantaris muscles (12 rats of each group) were processed by biochemical and histochemical analyses. Analyses of IGF-I and GH receptor mRNAs were performed at 3 d (5 rats of each group) and 14 d (5 rats of each group) after compensatory overload. Muscle weight and histochemical analyses (7 rats of each group) were performed at 14 d after compensatory overload.

Histochemistry. Seven rats of each group were sacrificed 14 d after compensatory overload for histochemical analysis. The plantaris muscle was quickly dissected and freed of any fat and connective tissues, with the wet weights being recorded. The plantaris muscle was then cut transversely at the 1/3 (distal) and 2/3 (proximal) points from the distal tendon and immediately frozen in isopentane cooled to -130°C with liquid nitrogen. Each tissue was cut into $10\text{-}\mu\text{m}$ -thick serial sections in a cryostat at -20°C . For the identification of muscle fiber types, tissue slices were processed by myofibrillar actomyosin ATPase after preincubation (room temperature) for 4 min at pH 4.3 and 4.6, and 10 min at pH 10.3 [19]. Muscle fibers were classified as type I, IIA, IIB, and IIC according to the nomenclature system of Brooke and Kaiser [20]. The areas of each fiber type were determined by counting ~60 fibers from the cross-sections at the 1/3 and 2/3 points from the distal tendon of the plantaris muscle. The measurements of fiber areas were carried

out using a Macintosh-based image analyzing system (NIH-image 1.54). Because only a minimal percentage of type IIA fiber in hypox rats was found, it was not included in the analysis of fiber area.

Analyses of IGF-I and GH receptor mRNAs.

Five rats of each group were sacrificed at 3 and 14 d after compensatory overload for analyses of IGF-I and GH receptor mRNAs. The plantaris muscle was quickly dissected and freed of any fat and connective tissues. It was then cut transversely at the 1/3 and 2/3 points from the distal tendon. The 1/3 distal part (distal) and 1/3 proximal part (proximal) of the plantaris muscle were immediately frozen in liquid nitrogen and stored at -80°C until used. Total RNA was extracted by the guanidium isothiocyanate-phenol-chloroform method described by Chomczynski and Sacchi [21]. RNA concentrations were determined spectrophotometrically at 260 nm. Ribonuclease protection assays of IGF-I and GH receptor mRNAs were carried out according to the method reported by Fiedberg *et al.* [22] using the Guadian RNase protection assay kit (Clontech, Palo Alto, CA, USA). ^{32}P radio-labeled 419 base pairs (bp) IGF-I mRNA and 376 bp GH receptor mRNA riboprobes were used. The riboprobe of GH receptor mRNA was complementary to 152 bp of a common extracellular region to GH-binding protein and GH receptor mRNAs, and to 120 bp of GH-binding protein mRNA specific hydrophilic tail. Thus a 272 bp fragment of the hybridization between the riboprobe and GH-binding protein mRNA as well as a 152 bp fragment of hybridization between the riboprobe and GH receptor mRNA were yielded as described by Fujikawa *et al.* [23]. Total RNA (40 μg) from the muscle was suspended in 30 μl hybridization buffer containing excess riboprobe. After hybridization at 37°C for 60 min, the samples were incubated with RNase A and T_1 at 37°C for 30 min. The RNases

were inactivated by digestion with proteinase K for 15 min, and the protected RNA hybrids were precipitated with 70% ethanol. The protected fragments were then denatured at 95°C for 3 min in the loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 10 mM EDTA) and electrophoresed in a 6% acrylamide-8 M urea gel. Bands corresponding to the probe fragments protected by IGF-I and GH receptor mRNAs were quantified using the NIH image 1.54 system. The data were expressed as a percentage of the value obtained from distal control in normal rats.

Statistics. Standard statistical procedures were employed to calculate the means and standard deviations. Two-way repeated ANOVA was used for the comparison between the groups. Differences between the means were regarded as significant at $p < 0.05$.

RESULTS

Body weights of hypox and normal rats 14 d after compensatory overload were 160.3 ± 8.02 and 252.3 ± 7.62 g, respectively.

Muscle weight and relative weight of plantaris 14 d after compensatory overload are shown in Fig. 1. Muscle weights of the plantaris in Normal-OL and Hypox-OL groups were significantly heavier than in Normal-Cont and Hypox-Cont groups, respectively (Fig. 1a). Relative weights of plantaris to body weight in Normal-OL and Hypox-OL groups were significantly greater than in Normal-Cont and Hypox-Cont groups, respectively (Fig. 1b). Muscle weights of the plantaris in Hypox-Cont and Hypox-OL groups were significantly lower than in Normal-Cont and Normal-OL groups, respectively (Fig. 1a). However, relative weights of plantaris to body weight in Hypox-Cont and Hypox-OL groups did not differ from those in

Fig. 1. Muscle weight (a) and the relative weight (b) of plantaris 14 d after compensatory overload. * $p < 0.05$ vs. Normal-Cont; † $p < 0.05$ vs. Hypox-Cont; ‡ $p < 0.05$ vs. Normal-OL.

Fig. 2. Muscle fiber areas of plantaris in normal rats 14 d after compensatory overload. * $p < 0.05$ vs. Distal-Cont; † $p < 0.05$ vs. Proximal-Cont; ‡ $p < 0.05$ vs. Proximal-OL.

Normal-Cont and Normal-OL groups, respectively (Fig. 1b).

Fiber areas of the plantaris muscle in normal rats 14 d after compensatory overload are shown in Fig. 2. Fiber areas of type I, IIA, IIB, and IIC in the distal part of Normal-OL group were significantly larger than Normal-Cont group (Fig. 2). Fiber areas of type I, IIA, and IIC in the proximal part of Normal-OL group were significantly larger than Normal-Cont group, however, the type IIB fiber area did not differ (Fig. 2). Fiber areas of the plantaris muscle in hypox rats 14 d after compensatory overload are shown in Fig. 3. Fiber areas of type I, IIB, and IIC in the distal part of Hypox-OL group were significantly larger than Hypox-Cont group (Fig. 3). Fiber areas of type I and IIC in the proximal part of Hypox-OL group were significantly larger than Hypox-Cont group, however, the type IIB fiber area did not differ (Fig. 3).

Figure 4 shows IGF-I mRNA expression in plantaris muscle at 3 (a) and 14 d (b) after compensatory overload. IGF-I mRNA expressions in the distal and proximal parts in Normal-OL group were significantly greater than in Normal-Cont group at 3 d after the

Fig. 3. Muscle fiber areas of plantaris in hypox rats 14 d after compensatory overload. * $p < 0.05$ vs. Distal-Cont; † $p < 0.05$ vs. Proximal-Cont.

compensatory overload (Fig. 4a), but at 14 d, IGF-I mRNA expressions in the distal and proximal parts in the Normal-OL group conversely were significantly decreased in comparison with in Normal-Cont group (Fig. 4b). IGF-I mRNA expressions in the distal and proximal parts in Hypox-OL group were significantly greater than in Hypox-Cont group 3 and 14 d after the compensatory overload (Fig. 4a, b). The IGF-I mRNA expressions in distal parts in Normal-OL and Hypox-OL groups 3 d after the compensatory overload were significantly greater than IGF-I mRNAs in the proximal parts, respectively (Fig. 4a).

Figure 5 shows GH receptor mRNA and GH-binding protein mRNA expressions in plantaris muscle 3 d after compensatory overload. GH receptor mRNA and GH-binding protein mRNA expressions in the proximal parts in normal and hypox rats were significantly greater than those in the distal parts, respectively. GH receptor mRNA and GH-binding protein mRNA expressions in the distal and proximal in Normal-OL group were significantly lower than in Normal-Cont group. GH receptor mRNA and GH-binding protein mRNA expressions in the distal and proximal parts in Hypox-OL group were significantly lower than in Hypox-Cont group (Fig. 5).

Fig. 4. IGF-I mRNA expression in plantaris muscle at 3 (a) and 14 d (b) after compensatory overload. The data were expressed as a percentage of the value obtained from distal control in normal rats. * $p < 0.05$ vs. Distal-Cont in normal or hypox rats; † $p < 0.05$ vs. Proximal-Cont in normal or hypox rats; ‡ $p < 0.05$ vs. Proximal-OL in normal or hypox rats.

GH receptor mRNA and GH-binding protein mRNA expressions in plantaris muscle at 14 d after compensatory overload are shown in Fig. 6. GH receptor mRNA expression in the proximal parts in Normal-OL was significantly greater than that in the distal part, however, GH receptor mRNA and GH-binding protein mRNA expressions in the proximal parts in hypox rats were decreased or unchanged, in comparison with those in the distal parts, respectively. GH receptor mRNA and GH-binding protein mRNA expressions in the distal and proximal in Normal-OL group were significantly lower than in Normal-Cont group. GH receptor mRNA and GH-binding protein mRNA expressions in the distal and proximal parts in Hypox-OL group were almost disappeared (Fig. 6).

DISCUSSION

This study found the degree of compensatory-overload-induced hypertrophy in plantaris muscle to be independent of hypophysectomy. Also, IGF-I mRNA expressions in the distal and proximal parts of plantaris muscle in normal and hypox rats were significantly increased at 3 d after the compensatory over-

Fig. 5. GH binding protein mRNA and GH receptor mRNA expressions in plantaris muscle at 3 d after compensatory overload. The data were expressed as a percentage of the value obtained from distal control in normal rats. * $p < 0.05$ vs. Distal-Cont in normal or hypox rats; † $p < 0.05$ vs. Proximal-Cont in normal or hypox rats; ‡ $p < 0.05$ vs. Proximal-OL in normal or hypox rats.

load. These results suggest that compensatory hypertrophy of plantaris muscle is independent of pituitary GH, and that IGF-I's independence of expression from pituitary GH plays an important role in compensatory hypertrophy. These observations support the previous reports that found GH-independent IGF-I to be of importance in muscle hypertrophy [13, 14].

In this study, the area of type IIB fiber in the proximal parts of plantaris muscle in normal and hypox rats did not change after compensatory overload. However, all the fiber areas measured in the distal parts of normal and hypox rats increased significantly, showing that hypertrophy in the distal part of the plantaris muscle following compensatory overload exceeds that in the proximal part. Region-specific hypertrophy in a muscle has been reported in studies on exercised humans [5] and functionally overloaded animals [4]. A possible explanation for the region-specific hypertrophy following compensatory overload is that the muscle fibers of the different parts of a muscle are recruited at different levels for various movements. Distal fibers in lateral gastrocnemius muscle may be mainly recruited for slow stepping exercise, whereas proximal fibers may be recruited in equal or greater amount for moderate to fast stepping exercise [6].

Fig. 6. GH binding protein mRNA and GH receptor mRNA expressions in plantaris muscle at 14d after compensatory overload. The data were expressed as a percentage of the value obtained from distal control in normal rats. * $p < 0.05$ vs. Distal-Cont in normal or hypox rats; † $p < 0.05$ vs. Proximal-Cont in normal or hypox rats; ‡ $p < 0.05$ vs. Proximal-OL in normal or hypox rats.

Muscle fibers along the proximal-distal axis in a muscle have different recruitment patterns [7]. Another explanation for the greater hypertrophy in the distal part of the plantaris muscle is that it may result from the change in the recruitment pattern in relation to the operating procedure used to induce the compensatory overload. Such procedure in this study was the removal of the distal half of the gastrocnemius and soleus muscles. We directly observed in the dissection that after the removal of the distal half, the remaining gastrocnemius and soleus muscles bonded to the proximal part of the plantaris muscle. Consequently, whereas the proximal part of plantaris muscle contracts with the bonded gastrocnemius and soleus muscles, the distal part of the plantaris muscle contracts independently. This difference may have caused region-specific hypertrophy of the plantaris muscle after compensatory overload in this study.

IGF-I mRNA expressions in the distal and proximal parts of the plantaris muscle were observed to be increased at 3 d after compensatory overload regardless of hypophysectomy. Also, in the distal part the increase in IGF-I mRNA expression was greater following compensatory overload. These results show that IGF-I mRNA expression following compensatory

overload differs between the parts of a muscle, and is dependent on the hypertrophy levels in a muscle. It has been reported that IGF-I mRNA expression increases during compensatory hypertrophy [13, 14], and that the suppressed growth of skeletal muscle in hypophysectomized rats is restored by the administration of IGF-I [8, 9]. These observations suggest that local IGF-I mRNA expression may be associated with the region-specific hypertrophy in a muscle that is induced by compensatory overload.

At 14 d after compensatory overload, IGF-I mRNA expressions in the distal and proximal parts increased in hypophysectomized rats, but decreased in normal rats. Thus a temporal discrepancy between IGF-I mRNA levels and muscle hypertrophy at 14 d after compensatory overload was observed in this study. It has been known that the changes of several mRNAs were transient and preceded the tissue growth [24, 25]. Such transience and precedence may explain the temporal discrepancy between IGF-I mRNA levels and the muscle hypertrophy.

Although the functions of GH receptor and GH-binding protein in skeletal muscle remain unclear, the existence of GH receptor in skeletal muscle is thought to be evidence of direct action by GH on skeletal muscle growth. Isgaard *et al.* [12] reported that GH receptor mRNA expression increased the volume of overloaded cardiac muscle. Jennische and Andersson [16] showed that the expression of GH receptor mRNA increases in skeletal muscle that is regenerating after ischemic damage. However, GH receptor mRNA and GH-binding protein mRNA expressions in this study were seen to have largely disappeared at 14 d after compensatory overload. Compensatory overload in this study induced region-specific hypertrophy of the plantaris muscle regardless of hypophysectomy. Muscle hypertrophy following compensatory overload occurs in GH-deficient hypophysectomized rats [3, 8, 14], and GH-independent IGF-I plays an important role in hypertrophy [13, 14]. These observations suggest that GH receptors in compensatory overloaded muscle may be unrelated to the hypertrophy, whereas GH receptors in the regenerating skeletal muscle or in cardiac muscle are related to the hypertrophy.

GH receptor mRNA and GH-binding protein mRNA expressions in the proximal parts in normal and hypox rats were greater than those in the distal parts at 3 d after compensatory overload. Also, at 14 d after compensatory overload in normal rats, the expression of GH receptor mRNA in the proximal part was higher than in the distal part. The direct action of GH on skeletal muscle has not been fully confirmed. However, these observations suggest that GH sensitiv-

ity in the plantaris muscle differs between the distal and proximal parts, and that GH-related muscle growth in the proximal part is greater than in the distal part. Williams and Goldspink [26] reported that the longitudinal growth of skeletal muscle results mainly from the newly formed sarcomere at the distal end. The findings of their report suggest that the region-specific expression of GH receptor mRNA observed in this study has a physiological function.

Whereas GH receptor mRNA and GH-binding protein mRNA expressions in the proximal parts at 3 d after compensatory overload were higher than those in the distal part, those in the proximal parts at 14 d after compensatory overload in hypox rats were decreased or unchanged as compared with those in the distal part. Also, at 14 d after compensatory overload in Normal-Cont, GH-binding protein mRNA in the proximal part was lower than in the distal part. We could not account for all these discrepant results. However, at 14 d after compensatory overload in hypox rats, GH receptor mRNA and GH-binding protein mRNA expressions were extremely weak. This indicates that hypophysectomy at 14 d after compensatory overload has a large effect on GH receptor mRNA and GH-binding protein mRNA expressions. Consequently, it is thought that the large effect of hypophysectomy modified the expressions of GH receptor and GH-binding protein mRNAs in distal and proximal parts. Also, if different sensitivities for hypophysectomy exist in distal and proximal parts, this may also influence the expressions of GH receptor and GH-binding protein mRNAs.

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