Slow-Twitch Myofibers are Observed in Masseter Muscle of the Hypothalamic Obese Rat

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(Received November 9, 1992)

Abstract The composition of the myofiber types classified immuno- and enzyme-histochemically were investigated in 12 muscles (*M. masseter*, *M. trapezius*, *M. triceps brachii*, *M. extensor carpi radialis*, *M. pectoralis profundus*, *M. longissimus*, *M. Biceps femoris*, *M. semitendinosus*, *M. semimembranosus*, *M. extensor digitorum longus*, *M. soleus* and *M. gastrocnemius*) of the obese rats with hypothalamic lesions operated at 25-28 day-old. The cross sectional area of each myofiber type was measured using automatic image analyzer. At 90 days after the operation, in the sham operated control animals (10 male rats), the masseter muscle was uniformly composed of fast-twitch type myofibers (Type IIA and IIB), which reacted for anti-fast-myosin monoclonal antibody and for alkali-stable myosin adenosine triphosphatase (ATPase) and were unreactive for acid-stable myosin ATPase. In the operated obese animals (10 male rats), however, the masseter muscle had not only fast-twitch myofibers but also slow-twitch myofibers (Type I), which reacted strongly for acid-stable myosin ATPase and were not reactive for anti-fast-myosin monoclonal antibody or for alkali-stable myosin ATPase, and showed a hypertrophy of myofibers. In comparison with sham-control rats, no change of histochemical fiber type composition was observed in other 11 muscles. All fiber types of these 11 muscles of the obese group were smaller in cross-sectional area than those of the sham-control group myofibers. It is suggested that the difference of the fiber type composition between the obese rats and the control rats indicates the transformation of the myofiber types from the fast-twitch type into slow-twitch type. It is considered that an adaptation to long-term jaw movements in the masseter muscle of the obese rats may lead to changes in composition of myofiber types.

Anim. Sci. Technol. (Jpn.) 64 (5) : 440-447, 1993

Key words : myofiber type, hypothalamic obese rat, histochemistry, morphometry

In mammals the hypothalamus contains very important sites controlling food intake. The bilateral lesions in the region of the ventromedial nuclei cause hyperphagia and bring about extreme obesity in the rat^{6,7)}. Such hypothalamic obese rats are noticed by many investigators in various research fields, because these can be easily produced by surgical and electrolytical means with an aid of stereotaxic instrument and change physiologically, endocrinologically, pharmacologically, morpho-

logically and ecologically^{2,3,8,9,22,30)}. However, no studies on the histochemical characteristics of myofiber in the obese rats with hypothalamic lesions have been reported yet. The aim of the present study, therefore, is to demonstrate the histochemical properties of the myofibers in 12 muscles of the obese rats.

Materials and Methods

Twenty male Wistar rats, 25-28 days of age, were used. The animals were maintained in a

room at $23 \pm 2^\circ\text{C}$ in which the light cycle was artificially controlled (12 h light, 12 h dark), and were provided food (rat chow) and water *ad libitum*. They were divided randomly into 2 groups. Ten rats (obese group) received electrolytic lesions in the ventromedial region of the hypothalamus under an anesthesia with sodium pentobarbital (5 mg/kg. i.p), and the remaining 10 rats (sham-control group) received sham-operation. The method of the operation was described in previous papers^{8,9}.

At 90 days after the operation, the animals were sacrificed by decapitation under ether anesthesia. Twelve muscles (M. masseter, M. trapezius, M. triceps brachii (Caput laterale), M. extensor carpi radialis, M. pectoralis profundus, M. longissimus thoracis, M. Biceps femoris (Caput longum), M. semitendinosus, M. semimembranosus, M. extensor digitorum longus, M. soleus and M. gastrocnemius (Caput laterale)) were carefully removed. The muscle samples (4–5 mm thick) sliced transversely in the midbelly region were mounted on a filter paper, and then were frozen in a mixture of dry ice and isopentane. Serial transverse sections (6 μm) were cut on a cryostat. Unfixed sections were incubated for histoenzymatical demonstration of myosin ATPase, succinate dehydrogenase (SDH), nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) and α -glycogen phosphorylase (PhR) activities as previously reported^{16–19}. The frozen sections were also stained immuno-histochemically using monoclonal anti-fast myosin antibody (MY-32 clone, Sigma Chem. Co., St. Louis, USA), which was produced using rabbit fast-myosin (type II myosin) as immunogen. The immunohistochemical method employed in this study were as follows. The frozen sections were fixed in acetone cooled at -80°C for 5 min and then were placed in 100% methanol with 0.3% hydrogen peroxide for 5 min at room temperature to block endogenous peroxidase activity. They were treated with 0.1% normal goat serum for 30 min at room temperature.

The primary monoclonal antibody, which was diluted in 0.1% bovine serum albumin, 0.01 M phosphate buffered saline (PBS, pH 7.4) to produce a 1/400 solution, was incubated with the sections for 18 hr at 4°C . After washing in PBS, the sections were incubated with biotinylated goat anti-mouse immunoglobulin and peroxidase-conjugated streptavidin from Vector Laboratories (Burlingame, USA) for 60 min at room temperature. The sections were again washed with three changes of PBS. The antigen-antibody complex was subsequently visualized by immersion in substrate solution (0.05% 3–3'-diaminobenzidine tetrachloride, 0.01% H_2O_2 , 0.05 M Tris-HCl, pH 7.2) for 5 min at room temperature. Control sections were incubated with normal mouse serum. Myofibers were classified into types I, IIA and IIB by differences in reactivity for these enzymes and antibody.

The photomicrographs were taken of cross sections stained with anti-fast myosin antibody and NADR-TR to determine the percentage of myofiber types and to measure the cross-sectional area. A total of 500–600 fibers per muscle section were classified and examined. Cross-sectional areas of 100–150 fibers in each myofiber type per muscle section were measured using automatic image analyzer system (BIOCOM-2000, Biocom, Paris, France).

Paired Student's *t* test was used to evaluate the significance of any differences in means between the groups. Differences between means were considered significant at $P < 0.05$. Each value represented mean $\pm$ S.D..

Results

As shown in Table 1, myofibers were categorized into types I (slow-twitch-oxidative), IIA (fast-twitch-oxidative-glycolytic) and IIB (fast-twitch glycolytic). In type I fibers negative reaction against anti-fast myosin antibody and high activities of acid-stable myosin ATPase, SDH and NADH-TR were observed. In type IIA fibers positive reaction against

Table 1. Histochemical characteristics of myofiber types in male rat muscles

Myofiber type	Anti-fast myosin antibody	Acid-stable myosin ATPase ¹	Alkali-stable myosin ATPase ²	SDH	NADH-TR	PhR
I	Negative	### ³	—	###	##	-/+
II A	Positive	—	###	##/###	##/###	##
II B	Positive	—	###	+	+	##/###

¹Preincubation at pH 4.3.²Preincubation at pH 10.5.³Enzyme activities: —, Unreactive; +, weak; ##, strong; ###, very strong.

anti-fast myosin antibody and high activities of alkali-stable myosin ATPase, SDH and NADH-TR were observed. In type IIB fibers positive reaction against anti-fast myosin antibody and high activities of alkali-stable myosin ATPase, and PhR were observed.

There was a significant difference in the obesity index²¹ between the obese group (0.412 ± 0.009) and the sham-control group (0.311 ± 0.003) ($P < 0.01$).

Composition of myofiber types was shown in Table 2. In sham-control group, the masseter muscle has types IIA and IIB fibers (61.4 ± 3.9 and $38.6 \pm 3.9\%$, respectively), but no type I fiber was present (Fig. 1). In obese group, however, the masseter muscle contained types I, IIA and IIB (8.2 ± 1.6 , 66.6 ± 3.7 and $25.2 \pm 3.8\%$, respectively) (Fig. 2). These slow-twitch (type I) myofibers were dominantly found in the deepest part of the masseter muscle. In other 11 muscles studied, there was no significant difference in myofiber type composition between the two groups.

Cross-sectional area of myofiber types was also given in Table 2. In the masseter muscle, types IIA and IIB fibers of obese rats (2568 ± 160 and $3231 \pm 174 \mu\text{m}^2$, respectively) were significantly ($P < 0.05$) larger in cross-sectional area than type IIA and IIB fibers of sham-control rats (2347 ± 186 and $2828 \pm 162 \mu\text{m}^2$, respectively). In obese group, the cross-sectional area of type I fibers ($2354 \pm 236 \mu\text{m}^2$) was smaller than that of types IIA and IIB fibers. However, all myofiber types in other

11 muscles of the obese rats were smaller in cross-sectional area than those of the sham-operated rats.

Discussion

It was clear from the obesity indexes of obese and sham-control groups that the operation of the electrolytic lesion in the ventromedial region of hypothalamus was successful in the present study²¹.

The percentages and cross-sectional areas of histochemical myofiber types in the 12 muscles of sham-control group were similar to those reported previously^{1,14,21,25-28,31}. Except for the masseter muscle, the myofiber type populations in the remaining 11 muscles of the obese rats also similar to those of the previous articles^{1,14,21,25-28,31}. Previous reports^{27,28} demonstrated that the masseter muscle was uniformly composed of fast-twitch (type IIA and IIB) myofibers. However, the masseter muscle in the obese rats contained not only fast-twitch myofibers (type IIA and IIB fibers) but also slow-twitch myofibers (type I fibers). With the exception of the masseter muscle, the cross-sectional areas in the remaining 11 muscles of the obese rats were smaller than those of sham-operated rats. The cross-sectional area in the masseter muscle of the obese group was significantly larger than that of the sham-control group. That is to say, the fiber type composition and myofiber size of the rat masseter muscle changed after the operation of the electrolytic hypothalamus lesions.

Myofiber Characteristics in the Obese Rat

Table 2. Comparison of the percentage and cross sectional area of myofiber types in twelve muscles between the obese and sham-control rats

Muscle	Fiber type	Percentage (%)		Area ¹ (μm^2)	
		Sham-control	Obese	Sham-control	Obese
Masseter	I	—	8.2 $\pm$ 1.6	—	2354 $\pm$ 236
	IIA	61.4 $\pm$ 3.9	66.6 $\pm$ 3.7	2347 $\pm$ 136	2568 $\pm$ 160
	IIB	38.6 $\pm$ 3.9	25.2 $\pm$ 3.8	2828 $\pm$ 162	3213 $\pm$ 174
Trapezius	I	3.6 $\pm$ 0.8	3.8 $\pm$ 1.3	2161 $\pm$ 267	2106 $\pm$ 202
	IIA	61.4 $\pm$ 3.9	66.6 $\pm$ 3.7	2899 $\pm$ 323	2743 $\pm$ 246
	IIB	35.0 $\pm$ 4.2	29.6 $\pm$ 3.0	3929 $\pm$ 156	3739 $\pm$ 117
Triceps brachii	I	4.0 $\pm$ 1.3	4.2 $\pm$ 1.5	1817 $\pm$ 132	1626 $\pm$ 151
	IIA	33.4 $\pm$ 3.1	32.8 $\pm$ 3.3	2058 $\pm$ 359	1937 $\pm$ 201
	IIB	62.6 $\pm$ 2.9	63.0 $\pm$ 4.0	4104 $\pm$ 299	3973 $\pm$ 263
Extensor carpi radialis	I	5.4 $\pm$ 0.8	5.2 $\pm$ 1.8	1196 $\pm$ 270	1107 $\pm$ 115
	IIA	45.2 $\pm$ 2.5	45.6 $\pm$ 2.9	1673 $\pm$ 173	1540 $\pm$ 160
	IIB	49.4 $\pm$ 3.0	49.2 $\pm$ 4.1	4756 $\pm$ 212	4566 $\pm$ 211
Pectoralis profundus	I	8.6 $\pm$ 2.2	8.4 $\pm$ 1.8	2433 $\pm$ 142	2208 $\pm$ 192
	IIA	67.8 $\pm$ 3.6	69.2 $\pm$ 4.1	3099 $\pm$ 162	2783 $\pm$ 166
	IIB	23.6 $\pm$ 5.8	22.4 $\pm$ 4.0	3892 $\pm$ 182	3799 $\pm$ 196
Longissimus thoracis	I	8.8 $\pm$ 2.5	8.6 $\pm$ 2.7	1929 $\pm$ 260	1893 $\pm$ 232
	IIA	58.4 $\pm$ 2.9	56.8 $\pm$ 3.3	2814 $\pm$ 203	2631 $\pm$ 344
	IIB	32.8 $\pm$ 4.4	34.6 $\pm$ 4.1	3991 $\pm$ 249	3838 $\pm$ 276
Biceps femoris	I	2.6 $\pm$ 1.3	3.0 $\pm$ 1.0	1922 $\pm$ 209	1766 $\pm$ 260
	IIA	29.8 $\pm$ 2.9	30.4 $\pm$ 2.9	2294 $\pm$ 478	2123 $\pm$ 201
	IIB	67.6 $\pm$ 2.8	66.6 $\pm$ 2.9	4227 $\pm$ 291	4071 $\pm$ 410
Semitendinosus	I	7.8 $\pm$ 0.6	8.4 $\pm$ 0.9	1689 $\pm$ 298	1613 $\pm$ 211
	IIA	47.6 $\pm$ 2.7	47.2 $\pm$ 1.9	1960 $\pm$ 203	1904 $\pm$ 262
	IIB	44.6 $\pm$ 2.8	44.4 $\pm$ 2.1	4848 $\pm$ 328	4761 $\pm$ 267
Semimembranosus	I	5.6 $\pm$ 1.3	5.2 $\pm$ 1.6	2556 $\pm$ 613	2435 $\pm$ 383
	IIA	36.8 $\pm$ 2.6	37.2 $\pm$ 1.9	3760 $\pm$ 451	3562 $\pm$ 363
	IIB	58.0 $\pm$ 2.8	57.6 $\pm$ 2.4	6093 $\pm$ 412	5960 $\pm$ 296
Extensor digitorum longus	I	5.0 $\pm$ 1.9	4.6 $\pm$ 1.1	1231 $\pm$ 327	1159 $\pm$ 147
	IIA	45.6 $\pm$ 2.2	44.4 $\pm$ 2.4	1743 $\pm$ 132	1604 $\pm$ 109
	IIB	49.4 $\pm$ 3.6	51.0 $\pm$ 2.7	4943 $\pm$ 456	4714 $\pm$ 378
Soleus	I	85.0 $\pm$ 1.7	85.8 $\pm$ 1.9	4023 $\pm$ 201	3871 $\pm$ 218
	IIA	15.0 $\pm$ 1.7	14.2 $\pm$ 1.9	3781 $\pm$ 264	3636 $\pm$ 237
	IIB	—	—	—	—
Gastrocnemius	I	8.4 $\pm$ 1.3	9.0 $\pm$ 1.6	2401 $\pm$ 353	2299 $\pm$ 222
	IIA	28.0 $\pm$ 2.2	28.4 $\pm$ 1.8	2443 $\pm$ 466	2141 $\pm$ 191
	IIB	63.6 $\pm$ 2.7	62.6 $\pm$ 2.6	4236 $\pm$ 441	4105 $\pm$ 221

Each value represents mean $\pm$ S.D. ¹Cross sectional area of myofibers.

It was reported that the change of slow-twitch myofibers into fast-twitch myofibers occurred as a result of brief strengthening exercise in the rat soleus muscle, whereas the change of fast-twitch myofibers into slow-twitch myofibers occurred as a result of low intensive-prolonged duration exercise in the soleus muscle⁹⁾. The percentages of myofiber types also changed after endurance training in rat hind limb muscles²¹⁾. Recently, histo-

Fig. 1. Light micrographs of the masseter muscle of adult male Wistar rats with sham operation. Serial sections ($6\mu\text{m}$ thick) of the muscle are stained for antifast-myosin monoclonal antibody (a) and for acid-stable myosin ATPase preincubated at pH 4.3 (b). Only fast-twitch myofibers are demonstrated. No slow-twitch myofiber is present. $\times 120$

Fig. 2. Light micrographs of the masseter muscle of adult male Wistar rats with hypothalamic lesions. The muscle tissue sections are stained for anti-fast-myosin monoclonal antibody (a) and for acid-stable myosin ATPase preincubated at pH 4.3 (b). The masseter muscle contained both slow-twitch and fast-twitch myofibers. $\times 120$

chemical fiber composition changed in rat soleus and gastrocnemius muscles after hypo- and hyperthyroidism treatment¹⁵⁾. In Japanese quail anterior latissimus dorsi muscle, which is unique amongst vertebrates since it has been

considered to be made up by true slow fibers, fast myosin positive myofibers with hypertrophy were demonstrated immunohistochemically after prolonged stretch²⁰⁾. This finding revealed the myofiber type transformation

from the slow-twitch type into fast-twitch type in avian skeletal muscle. Our result demonstrates the transformation of the histochemical myofiber types from the fast-twitch type into slow-twitch type in mammalian skeletal muscle. Many factors, *i.e.* stretch²⁰⁾, exercise^{4,12,21)}, surgical and functional overload^{11,29)}, denervation^{13,23)}, reinnervation^{5,13)}, neuron secreting substances²⁴⁾, thyroid and sex hormones^{5,15)}, *etc.*, contribute the transformation of the muscle fiber types. However, the molecular mechanism of the myosin isoform transition is undefined.

Endocrinological and ecological properties of the hypothalamic obese rats were different from those of the control rats^{2,3,6-10,22,30)}. If endocrinological change in the obese rats played a causative role in the transformation of myofiber type, the fiber composition in most of the muscle studied should change as in case of hypo- and hyperthyroidism rats¹⁵⁾. Food intake time was increased about threefold in rats with hypothalamic lesions^{7,8,10)}, so it was considered that duration of jaw movement in eating was longer in the obese rats than in the control rats. This prolonged jaw movement in the obese rats may lead to the changes of immunohistochemical and histochemical characteristics of the masseter myofibers. It is suggested that the difference of the fiber type composition between the obese rats and the control rats indicates the transformation of myofiber types.

Acknowledgement

The authors wish to express our deep gratitude to Drs. E. SATO, J-A. GRIMAUD and C. PORTO for their valuable suggestions regarding the hypothalamic obese operation and morphometrical analysis, and also thank Mr. K. MATSUMOTO for his skillful operation assistance.

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視床下部性肥満ラットの咬筋に遅筋線維が観察される

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各 10 匹のラットに視床下部満腹中枢破壊手術および擬手術を 25~28 日齢で施した。手術後 90 日の肥満ラットおよび擬手術ラットの 12 種の骨格筋（咬筋，僧帽筋，上腕三頭筋，橈側手根伸筋，深胸筋，最長筋，大腿二頭筋，半腱様筋，半膜様筋，長指伸筋，ヒラメ筋および腓腹筋）における免疫および酵素組織化学的筋線維型の構成を調べた。各筋線維型別に筋線維の横断面積を画像解析装置を用いて計測した。擬手術ラットの咬筋は，速ミオシン抗体に陽性でアルカリ安定ミオシン ATPase 活性が高く酸安定ミオシン ATPase 活性の低い速筋線維（IIA および IIB 型）のみから構成されていた。しかし，視床下部性肥満ラットの咬筋においては，これらの速筋線維以外に，速ミオシン抗体に陰性でアルカリ安定ミオシン ATPase 活性が低く酸安定ミオシン ATPase 活性の高い遅筋線維（I 型）が観察され，かつ筋線維の肥大が認められた。両処理群間で，他の 11 種の骨格筋の筋線維型構成の有為差は認められなかったが，視床下部性肥満ラットの筋線維の横断面積は全ての筋線維型で擬手術ラットより小さかった。以上の結果から，速筋線維から遅筋線維へ筋線維型が変換することが判明した。この変換は，視床下部性肥満ラットの咬筋に運動強度は弱いが長期に及ぶ運動負荷がかかったことに起因すると考えられた。

日畜会報, 64 (5) : 440-447, 1993