Muscle Myoglobin as Determined by Electrophoresis in Thermally Acclimated Rat

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Abstract Quantitative analysis of myoglobin (Mb) was established using the sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis in order to determine the influence of thermal acclimation on Mb levels in muscles. Myoglobin was measured in the red and white parts of quadriceps, soleus, diaphragm and heart of young adult male rats (11 weeks old). It was higher in the red muscle (0.92 ± 0.043 mg/g fresh weight) than in the white muscle (0.08 ± 0.007), and similar in the red muscle and soleus (1.17 ± 0.117). Heart showed the highest level (1.52 ± 0.073) among the tissues studied. The level in diaphragm (0.74 ± 0.039) was intermediate between red muscle and heart. Cold acclimation (5°C for 4 weeks, 11 weeks old) caused significant increases in Mb levels in white muscle (0.27 ± 0.031 , $p < 0.001$), heart (1.95 ± 0.094 , $p < 0.01$), and diaphragm (1.01 ± 0.060 , $p < 0.01$), but not in red muscle and soleus. The rats reared in cold for many generations (20 generations at 5°C , 11 weeks old) manifested significant increases in Mb levels of all tissues ($p < 0.05$ – 0.001) examined. Heat acclimation (33°C for 4 weeks, 11 weeks old) did not influence Mb levels of the tissues. The above findings suggest that skeletal muscle Mb may be partly involved in an enhanced thermogenesis in cold acclimation by favouring an oxidative capacity of muscles.

Key words: myoglobin, gel electrophoresis, cold acclimation, heat acclimation, skeletal muscle.

Brown adipose tissue has been claimed to be a major site of nonshivering thermogenesis, and this has been intensively investigated (NICHOLLS and LOCKE, 1984). Although the brown adipose tissue is a principal effector of the increased nonshivering thermogenesis during cold acclimation, other tissues than brown adipose tissue could not be completely excluded. Especially, skeletal muscle, by

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virtue of its large mass, has been regarded as a potential candidate for an alternative significant site of nonshivering thermogenesis. In fact, skeletal muscle has been shown to undergo histological, histochemical, and physiobiochemical changes in cold-acclimated animals that may be related to an enhanced capacity for heat production. Cold acclimation caused the increased total volume (BUSER *et al.*, 1982), number (BEHRENS and HIMMS-HAGEN, 1977; YAHATA and KUROSHIMA, 1977) and modified size (BEHRENS and HIMMS-HAGEN, 1977; YAHATA and KUROSHIMA, 1977) of skeletal muscle mitochondria. Oxidative (succinate dehydrogenase) and lipid (β -hydroxy butyrate dehydrogenase) enzymatic activities as assessed histochemically were shown to increase in the calf muscles of cold-acclimated rats (POLLOCK *et al.*, 1973), while glycolytic enzymes such as α -glycerophosphate dehydrogenase did not change. Skeletal muscles from the cold-acclimated rats also exhibited increased $\text{Na}^+ - \text{K}^+$ -ATPase (GUERNSEY and STEVENS, 1977) and lipoprotein lipase (BEGIN-HEICK and HEICK, 1977) activities. Cold exposure (JANSKÝ and HART, 1963) and noradrenaline infusion (JANSKÝ and HART, 1963; GRUBB and FOLK, 1976) stimulated oxygen consumption of the perfused hind limb of cold-acclimated rats.

Myoglobin (Mb) in the skeletal muscle is an important factor that controls an adequate oxygen supply to muscle mitochondria (WITTENBERG, 1970) and, therefore, any modification of Mb concentration is expected to occur to meet oxygen requirement if thermogenic capacity or energy need of skeletal muscle is changed. In fact, Mb concentration was reported to increase in the exercising animals or the animals rearing at hypoxia (WITTENBERG, 1970). Recent studies, however, indicated that the exercise training did not increase skeletal muscle Mb level (MCDONALD *et al.*, 1984). It was also reported that Mb levels changed or did not change in the different muscles by exercise training (HARMS and HICKSON, 1983). Few studies have been reported so far about Mb in relation to cold acclimation. They showed significant increases in Mb levels of hind limb in mice, hamsters (CHAFFEE *et al.*, 1965), and that of heart in guinea pigs (BUI and BANCHERO, 1980) due to cold acclimation. In these studies Mb concentrations were measured by indirect spectrophotometric methods (REYNAFARJE, 1963; BUI and BANCHERO, 1980). The methods include the correction for hemoglobin present in the tissue assuming that coloured substances other than Mb and hemoglobin, such as cytochromes, do not influence the assay. In the present study we have established the method of Mb determination which specifically isolates and quantifies Mb by gel electrophoresis and examined the effect of thermal acclimation on Mb concentration in different types of muscles; red and white muscles of the quadriceps, intermediate muscle of soleus, heart, and diaphragm.

METHODS AND MATERIALS

Animals. Male Wistar rats (Shizuoka Laboratory Animal Center, Hamamatsu) were used in the study. At the start of the experiment the animals were 7 weeks old and weighed 180–190 g. All animals were placed under the artificial

lighting from 7:00 to 19:00, and provided an ad libitum diet of rat chow (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) and tap water. Warm controls were kept at $25 \pm 1^\circ\text{C}$ and relative humidity of ca. 50%. Cold-acclimated rats were exposed to $5 \pm 1^\circ\text{C}$ and heat-acclimated rats to $33 \pm 1^\circ\text{C}$ and relative humidity of ca. 30% continuously for 4 weeks. Rats reared in cold for 20 generations were also used in this study. Preparation of these animals was described elsewhere (MORIYA *et al.*, 1985).

Muscle sampling. The animals were decapitated and exsanguinated. The muscles chosen for the study were the fast twitch white muscle of superficial quadriceps with a low respiratory capacity, the fast twitch red muscle of deep quadriceps with a high respiratory capacity, the slow twitch red soleus with a moderately high respiratory capacity (WINDER and HOLLOSZY, 1977), heart and diaphragm. These muscles were rapidly dissected out and stored at -30°C until Mb analysis.

Mb determination. The minced muscle was homogenized in the ice-cold acetic acid buffer (0.01 M, pH 4.5, 19.25 mg/g tissue) using a Polytron homogenizer followed by further homogenization with a Potter Elvehjem homogenizer in the iced water bath. The homogenate was centrifuged for 15 min at 22,600 *g* at 4°C and the extract supernatant was used for Mb determination.

Polyacrylamide slab gel electrophoresis. Gel electrophoresis was carried out by the method of LAEMMLI (1970) using 15% gel. The Mb samples for electrophoresis were prepared by incubating the extract supernatant mentioned above at 60°C for 10 min with 10% sodium dodecyl sulfate (SDS) (2.65 ml), 2-mercaptoethanol (0.3 ml), and saturated bromophenol blue solution (0.05 ml) (*v/v* = 1/1). The electrode buffer (pH 8.4) contained 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. Thirty μl sample solution was applied per sample well with a microsyringe and the electrophoresis was carried out with a current of 20 mA until the bromophenol blue marker reached the bottom of the gel (usually 3 to 4 h). Staining of the proteins in the gel with Coomassie brilliant blue (CBB) and destaining the background gel were carried out as described previously (MURAKAMI and UCHIDA, 1985). The amount of Mb in a band was determined by quantification of the extracted CBB (FENNER *et al.*, 1975). The eluted dye was analyzed for absorbance at 605 nm using Hitachi 101 Spectrophotometer. Sperm whale Mb was used as standard, since its biophysical nature was well examined (KATZ and DENIS, 1970). A known concentration of sperm whale Mb, which was approximate to the tissue Mb level, was applied on all slab gels as an internal standard in order to correct any variations in staining from gel to gel.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed according to the method of O'FARREL *et al.* (1977) as modified by MURAKAMI and UCHIDA (1984). The Mb sample was prepared by adding 50 μl of solution 9.5 M urea/2.3% Nodinet P-40/2.3% 2-mercaptoethanol/2% ampholytes to 50 μl extract supernatant. Fifty μl of mixed solution was loaded onto first-dimensional cylindrical gel. Electrophoresis was carried out for 4 h at 400 V at room

temperature. The second slab gel electrophoresis was performed at 5 mA for about 3 h so that the tracking dye neared the bottom of the tracking gel, followed by 20 mA for about 4 h until the dye reached the bottom of the separating gel.

Statistics. Unpaired *t*-test was used to evaluate the difference. All results were expressed as mean $\pm$ standard error.

RESULTS

Identification and quantification of Mb

Figure 1 shows electrophoretogram of sperm whale Mb in various concentrations. Standard solutions of Mb were prepared on the basis of specific extinction coefficient of sperm whale Mb, 90.0 ($E_{1\text{cm}}^{1\%}$ at 409 nm) (KATZ and DENIS, 1970). Total absorbances (net absorbances multiplied by the volume of the pyridine used for elution of the dye) for these Mb bands were plotted against the amounts of Mb applied to the gel (Fig. 2), indicating a linear relation between total absorbances and amounts of Mb.

The actual gel pattern of muscle extract stained with CBB is presented in Fig. 3. The Mb bands, corresponding to the standard sperm whale Mb, were shown to be well separated. The purity of the isolated Mb was monitored by the two-dimensional gel electrophoresis with sperm whale Mb as shown in Fig. 4a-c. As a comparison, brown adipose tissue sample prepared similarly as muscle tissues was also run on the same slab gel in the second dimension, but no Mb spot was observed on the electrophoretogram (Fig. 4d).

Fig. 1. Electrophoretograms of sperm whale myoglobin (Mb) at various concentrations.

Fig. 2. Relationship between total absorbances and amounts of sperm whale myoglobin (Mb).

Table 1. Myoglobin (Mb) concentration in cold-acclimated rats.

Group	No.	Age (week)	Mb (mg/g fresh weight)				
			Red quadriceps	White quadriceps	Soleus	Heart	Diaphragm
WC	10	11	0.92 ± 0.043	0.08 ± 0.007	1.17 ± 0.117	1.52 ± 0.073	0.74 ± 0.039
CA-4wk	10	11	0.94 ± 0.046	0.27 ± 0.031^c	1.37 ± 0.196	1.95 ± 0.094^b	1.01 ± 0.060^b
C20G	12	11	1.57 ± 0.077^c	0.12 ± 0.012^a	1.69 ± 0.173^a	1.90 ± 0.063^c	1.33 ± 0.105^b

WC, warm controls; CA, cold-acclimated rats; C20G, rats reared in cold for 20 generations. *p* vs. WC: ^a <0.05, ^b <0.01, ^c <0.001.

Mb concentrations of the tissues

The results are summarized in Tables 1 and 2. In the warm control groups the highest Mb level was found in the heart and the lowest in the white quadriceps; Mb level of red quadriceps was more than ten times as high as that of white quadriceps. Soleus showed a comparable value of Mb to that of red quadriceps. Diaphragm had the intermediate level of Mb between those of red quadriceps and heart.

Fig. 3. Electrophoretograms of the samples of various muscles. a, sperm whale myoglobin (Mb) (0.503 μ g); b, sperm whale Mb (1.530 μ g); c, red quadriceps (warm controls); d, red quadriceps (reared in cold for 20 generations); e, white quadriceps (warm controls); f, white quadriceps (reared in cold for 20 generations); g, soleus (warm controls); h, soleus (reared in cold for 20 generations); i, heart (warm controls); j, heart (reared in cold for 20 generations).

Table 2. Myoglobin (Mb) concentration in heat-acclimated rats.

Group	No.	Age (week)	Mb (mg/g fresh weight)				
			Red quadriceps	White quadriceps	Soleus	Heart	Diaphragm
WC	10	11	1.00 $\pm$ 0.071	0.09 $\pm$ 0.012	1.14 $\pm$ 0.061	1.71 $\pm$ 0.133	0.95 $\pm$ 0.039
HA	10	11	0.86 $\pm$ 0.069	0.09 $\pm$ 0.011	1.14 $\pm$ 0.076	1.71 $\pm$ 0.102	0.91 $\pm$ 0.052

WC, warm controls; HA, heat-acclimated rats.

Cold acclimation for 4 weeks with full development of nonshivering thermogenesis (JANSKÝ, 1973) effected significant elevations of Mb levels in the white quadriceps, heart, and diaphragm, but not in red quadriceps and soleus (Table 1). In the rats reared in cold successively for 20 generations Mb levels of all the tissues examined were significantly higher than those in the warm controls of the same age

Fig. 4. Two-dimensional electrophoretograms of the samples. a, sperm whale myoglobin (Mb); b, white quadiceps; c, red quadiceps; d, brown adipose tissue.

(Table 1).

Mb levels of the muscles were not different in the heat-acclimated rats from those in the warm controls (Table 2).

DISCUSSION

Mb concentrations. The values of Mb levels in the present study were considerably lower as compared with those determined by the spectrophotometric method (REYNAFARJE, 1963; HARMS and HICKSON, 1983), but the heart Mb level was about two times higher than the value determined by the photometric method followed by chromatography of the sample on carboxymethylcellulose (MCDONALD *et al.*, 1984). The more direct approach with gel electrophoresis established in the present study may give a more accurate estimation of Mb in the tissues. The spectrophotometric method by REYNAFARJE (1963), most widely used for Mb, detected Mb activity in the brown adipose tissue (our unpublished result) which is known to be rich in other pigments such as mitochondrial cytochromes and hemoglobin in the vessels, while Mb spot was never found on the electrophoretogram of this tissue (Fig. 2).

It is well established that a high level of Mb is contained in a highly oxidative muscle with high activities of respiratory enzymes such as cytochrome oxidase and succinate dehydrogenase (HARMS and HICKSON, 1983; WINDER and HOLLOSZY, 1977). It was confirmed in the present study that the muscles with high respiratory capacity have higher Mb levels. It was noted that Mb level in the red muscle of quadriceps was about 10 times higher than that in the white muscle of quadriceps. A similar difference was found elsewhere (HARMS and HICKSON, 1983), although Mb values were different from those in this report as mentioned above.

Changes in Mb levels in the thermally acclimated rats. Cold acclimation caused about threefold increase in Mb level of white quadriceps, while no significant changes in those of red quadriceps and intermediate muscle of soleus (Table 1). These three muscle fiber types have been shown to respond differently to the same physiological stimulation such as exercise, thyroid hormones and cold. Mitochondrial α -glycerophosphate dehydrogenase increased in the red muscles, but did not change in the white muscle in the thyroid hormone-treated rats (JANSSEN *et al.*, 1978; WINDER and HOLLOSZY, 1977). The rat red quadriceps had the greater increases of mitochondrial enzymes such as citrate synthase and succinate dehydrogenase and also Mb concentration by training as compared with the white quadriceps and soleus (HARMS and HICKSON, 1983). Succinate dehydrogenase (DEBRIS and FILIPCHENKO, 1975) and lipoprotein lipase (BEGIN-HEICK and HEICK, 1977) activities were activated specifically in the red muscle fibers of cold-acclimated rats. Creatine level and its uptake of soleus, but not of white muscle, gastrocnemius, was decreased in the cold-acclimated rats (KURAHASHI and KUROSHIMA, 1978). These findings suggest an enhancement of oxidative thermogenesis in the more oxidative skeletal muscle by certain manipulations; site-specificity of muscular

metabolic modifications induced by cold acclimation as well as exercise and hormones. Moreover, it was suggested from histochemical study (succinate dehydrogenase) that the white muscle fibers became more like the red muscle fibers with higher oxidative enzyme activity by cold acclimation (BEHRENS and HIMMS-HAGEN, 1977). The elevated Mb level in the white muscle of cold-acclimated rats may, thus, reflect a transition of its metabolism to a more oxidative direction, contributing to an improved thermogenesis in the skeletal muscles as a whole. Myoglobin levels also increased in the heart and diaphragm by 27 and 36% in the cold-acclimated rats, respectively, possibly resulting from the enhanced circulatory and respiratory functions (JANSKÝ, 1973). Changes in body activity might have influenced the tissue Mb levels in the present study. Exercise training was reported to elevate Mb levels of the heart (MCDONALD *et al.*, 1984) and skeletal muscles (HARMS and HICKSON, 1983). However, it was reported that the amount of body activity was decreased in the cold-acclimated animals (BARRET and MOUNT, 1967). It is therefore very likely that the changes in Mb levels found in the present study were specific to cold acclimation. The present results accord with those reported in the gastrocnemius (white muscle) of cold-acclimated mice and hamsters (CHAFFEE *et al.*, 1965) and in the heart ventricles of cold-acclimated guinea pigs (BUI and BANCHERO, 1980). Significant increase of Mb levels was also reported in the diaphragm and gastrocnemius in Alaskan snowshoe hares during winter as compared with those during summer (ROSENMAN and MORRISON, 1965). As mentioned earlier, however, Mb concentrations in these studies were measured by the spectrophotometric method and not compared among the different muscles. As shown here, cold acclimation with enhanced nonshivering thermogenesis (JANSKÝ, 1973) caused significant elevation of Mb in the white muscle, but not in the red muscle. The result differed in site-specificity from the previous studies that exercise training or thyroid hormones produced an increase in Mb of red muscle, but not in the white muscle (HARMS and HICKSON, 1983; JANSSEN *et al.*, 1978; WINDER and HOLLOSZY, 1977). Accordingly, it is inferred that different mechanisms or factors are involved in the changes of Mb induced by cold acclimation as compared with exercise or thyroid hormones.

It was interestingly noted that Mb levels were more significantly elevated in all the tissues examined in the animals reared in the cold for many generations than in the warm controls of the same age (Table 1). These rats showed greater nonshivering thermogenesis than the usual cold-acclimated rats exposed to cold for periods of 2 to 8 weeks (MORIYA *et al.*, 1985). Moreover, it was shown that even the rats reared in the warm for 3 generations after being reared for many generations in the cold exhibited much more nonshivering thermogenic capacity as compared to the warm controls. Such lasting consistency of cold adaptability in rats reared in cold for many generations may be explained by genetic changes or selection in cold tolerance. In any case, the higher Mb levels observed in the animals reared in the cold for many generations may contribute to the more improved thermogenic capacity than that in the usual cold-acclimated ones.

Depressed nonshivering thermogenesis was found in the heat-acclimated

animals (PETROVIĆ and MARKOVIĆ-GIAJA, 1973). Lowered resting metabolism (ROUSSET *et al.*, 1984) and oxidative enzymes in the liver, but not in the kidney, heart and skeletal muscle (BEDRAK and SAMOILOFF, 1967) were also reported in the heat-acclimated rats. Any changes in Mb levels in the muscles were not obtained in the present study, suggesting that changes in muscle Mb are not related to the depressed metabolism, and tissues other than muscles are possibly involved.

Although no conclusive evidence in favour of a role for skeletal muscle in nonshivering thermogenesis yet exists, some indirect data have been presented as described in the Introduction, indicating that skeletal muscle may be involved in an enhanced thermogenesis induced by cold acclimation. The present findings on the changes in the Mb of skeletal muscles would also be another clue as to this problem.

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