Effects of Soft Diet on Rat Masseter Muscle Mitochondrial Development

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Summary: There is not fine information regarding the influence of diet on development of masseter muscle and its mitochondria. Objective of this study is to compare mitochondrial enzyme activity, and morphology of masseter muscle fiber cells and its mitochondria prepared from rats fed soft diet to those fed hard diet. Cross-sectional area of fiber cells and number of mitochondria per unit area prepared from rats fed hard diet were greater than those of animals fed soft diet on postnatal day 60, and these differences decreased under both feeding conditions on days 120. Structure of mitochondrial cristae of rat fed hard diet was clear but that of fed soft diet ambiguous and contains many halos. Mitochondrial succinate-O₂ and NADH-O₂ oxidoreductase activities isolated from rats fed hard diet were higher than those of soft diet group over the period from days 30, and differences in both diet groups became the largest on days 60, and decreased on days 120. Our results indicated that development of masseter muscle fiber cells and mitochondria is hindered when rats are fed soft diet, but recover partially later, and hard diet is required for normal development of masseter muscle.

Masseter muscle is of great importance for food intake in mammals. Normal development of the masseter muscle within the appropriate period is critical for the growth of mammals, and is partially dependent on the physical properties of food, such as hardness. Rats change their feeding behavior from suckling to mastication during the period from postnatal days 10 to 15¹⁶⁾. Several studies have indicated that feeding a soft diet induces significant decreases in the diameter and changes in other properties of rodent masticatory muscle fibers compared to the controls fed a normal diet after weaning^{7–9,11–14)}.

One of the most important roles of mitochondria in muscle is energy production for normal muscle function, which is associated with growth and development of the individual¹⁶⁾. In our previous study, we reported structural and numerical development of rat masticatory muscle mitochondria and changes in enzyme activity of the respiratory chain to characterize the molecular and cellular events

associated with the behavioral change from suckling to mastication¹⁶⁾. Our results indicated that the cross-sectional area of mitochondria increased between postnatal day 10 and 15 when feeding behavior changes from suckling to mastication. We also showed that the levels of activities of NADH-O₂ and succinate-O₂ oxidoreductases of the mitochondrial respiratory chain increased during the same period. In the present study, we compared the morphology of masseter muscle fiber cells and their mitochondria, and the mitochondrial respiratory chain enzyme activity in rats fed a soft diet and those fed a hard diet, after feeding behavior changed from suckling to mastication.

Materials and Methods

Light and transmission electron microscopy

All protocols conformed to the guidelines for the care and use on laboratory animals of Nippon

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Dental University, and were reviewed and approved by the Institutional Animal Care and Use Committee of our university. Control rats (Wistar rats, male, $n = 21$ for microscopy and $n = 60$ for activity assay) were fed a solid pellet diet (hard diet, MF, Oriental Yeast Inc., Tokyo, Japan), and experimental Wistar rats (male, $n = 21$ for microscopy and $n = 60$ for activity assay) were fed a fine grained diet (soft diet, MF powder, Oriental Yeast Inc., Tokyo, Japan). Rats were deeply anesthetized with ether and sacrificed by cervical dislocation. Small blocks of masseter muscle from rats at 0, 21, 26, 30, 35, 60, and 120 days postnatally ($n = 3$, on each day) were fixed in 2% glutaraldehyde-cacodylate buffer (pH 7.2) for 2 hr at 4°C, and postfixed in 1% osmic acid for 1 hr at 4°C. After washing with cacodylate buffer (pH 7.2), they were dehydrated in absolute ethyl alcohol, and then embedded in Epon 812. Complete cross-sections (1 μm thickness) were stained with toluidine blue, and observed under a light microscope (Vanox-S, Olympus Co., Tokyo, Japan). Cross and longitudinal thin sections of fibers for transmission electron microscopy were prepared with an ultramicrotome (Ultracut, Reichert-Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. A Hitachi H-700 transmission electron microscope operating at 80 kV was used for observations. Ten photographs (magnification, $\times 10,000$) were selected randomly from each section. The cross-sectional areas of muscle fiber and mitochondria, length of mitochondria, and number of mitochondria per unit area (number/ μm^2) of muscle fibers were measured or counted arbitrarily from several locations using a photo analyzer (Pias LA-500, Osaka, Japan) linked to a personal computer (NEC PC-9801, Tokyo, Japan). A total of 100 muscle fibers and 200 mitochondria in each field were measured. For the measurement of the rate of halo in mitochondria, 100 ultramicrographs of the muscle fiber of the masseter were randomly selected from each sample after photographing the magnified images ($\times 20,000$) of cross section of the mitochondria.

Isolation of mitochondria

Mitochondria were isolated by the method of Hogeboom (1955) with slight modifications⁵⁾. Immediately following the sacrifice of 0-(Wistar rats, male, $n = 20$), 21-($n = 10$), 30-($n = 10$), 35-($n = 10$), 60-($n = 5$), and 120-day-old rats ($n = 5$) under anesthesia, masseter muscles were collected and minced in ice-cold isolation fluid containing 0.1 mM EDTA, 0.25 M sucrose, and 5 mM Tris-HCl (pH 7.4). Each sample was homogenized by 10 strokes with a Teflon pestle at 1,000 rpm. The ho-

mogenate was diluted about 10 fold with isolation fluid and centrifuged at $700\times g$ for 10 min. The supernatant was centrifuged at $7,000\times g$ for 10 min. The resulting sediment consisted of an upper fluffy layer and lower heavy layer. The heavy layer was collected and washed once in isolation fluid and then twice in 0.25 M sucrose at protein concentrations from 0.5 to 1.0 mg/ml. The preparation of mitochondria was carried out at 4°C. The samples were stored at -70°C for later use.

Enzyme assays

Rat masseter muscle mitochondria (3.0–10 μg of protein) were used for each assay. Succinate- O_2 and NADH- O_2 oxidoreductase activity was measured polarographically using a Clark-type oxygen electrode (Yellow Spring Ltd., Ohio). The assay of oxidase activity with 50 mM Tris-HCl (pH 7.5) was performed at 25°C in a closed 2 ml reaction chamber equipped with a magnetic stirrer, and rate of oxygen uptake was monitored. Each reaction was initiated by the addition of substrate, succinate (10 mM) or NADH (500 μM). Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard¹⁰⁾.

Statistical analysis

Mann-Whitney's U-test was used to test the statistical difference of values obtained from the mitochondria or muscle fibers prepared from rat fed soft diet by comparison with those from rat fed hard diet.

Results

Previously, we showed the developmental changes of masticatory muscle of rats when feeding behavior altered from suckling to mastication¹⁶⁾. In the present study, we further examined the developmental differences after weaning when rats are fed a soft diet that is fine grained and easy to eat and therefore poses no resistance to the movement of masseter muscles, or hard diet as controls. The cross-sectional area of masseter muscle fiber cells increased markedly from day 21 to 26, stopped increasing until day 35, increased again until day 60, and finally stopped growing (Fig. 1a). Until day 35, there were no differences between the cross-sectional area of muscle cells of rats fed the soft or the hard diet. However, the average area of cells from rats fed the hard diet was about 1.9-fold greater than that in the soft diet group on day 60. The values of cross-sectional area of fiber cells from rats fed the soft diet were more similar to those in the hard diet group on day 120 (about 1.4-fold).

Fig. 1. Developmental changes in several measurements of mitochondria and muscle fibers of Rats. fed a hard diet (open circles) or soft diet (dots). a) cross-sectional area of muscle fiber, b) cross-sectional area of mitochondria, c) length of mitochondria, d) number of mitochondria per cross-sectional area of muscle fiber. Values are means and standard deviation. (n = 100 (a), n = 200 (b, c, d), **P < 0.01, *P < 0.05, Mann-Whitney's U-test)

Fig. 2. Micrographs of masseter muscle of rat. a–b) light micrographs of cross-sections of muscles of rats fed a hard diet (a, day 60) and a soft diet (b, day 60) (Bar = 20 μm).

Fig. 3. Electron micrographs of cross section of mitochondria in masseter muscle (Bar = 500 nm). a) day 0, b) day 21, c) day 26 in hard diet, d) day 26 in soft diet, e) day 30 in hard diet, f) day 30 in soft diet, g) day 35 in hard diet, h) day 35 in soft diet, i) day 60 in hard diet, j) day 60 in soft diet, k) day 120 in hard diet, l) day 120 in soft diet.

We then investigated the morphology of mitochondria in fiber cells. The length and area of mitochondria increased transiently, and the major peaks were observed between day 30 and 35 (Fig. 1b, c). Both the values decreased on day 60, and remained at steady level until day 120. There were no marked differences in the time course of cross-sectional area or length of mitochondria between the rats fed the hard or the soft diet. The changes in

the number of mitochondria per unit area showed a very complicated pattern as shown in Fig. 1d. The number increased on day 21, then decreased on day 26, and gradually increased from day 30. The largest difference in number of mitochondria between rats fed the hard and soft diets was observed on day 60 (number in the hard diet group was 1.8-fold greater than that the soft diet group).

Light microscopy (Fig. 2a, b, on day 60) was

Fig. 3 (continued)

employed to analyze the differences in the morphology of fiber cells prepared from rats in the two diet groups; no differences were seen in fiber cells between the two groups except in their average size as described above (Fig. 1a). The transition electron microscopy showed the morphological development of mitochondria. Mitochondria are found aligned in myofibrils, clustered in the inner side of the muscle cytoplasmic inner membrane. The day 0 postnatal mitochondria are small, and undeveloped sparse tubular cristae are short and extended only

about half way across mitochondria (Fig. 3a). However, the inner membrane is shown to change from tubular to septate-like structure and to become more complex as the surface area of the inner membrane increases, and extend completely across mitochondria on day 21 (Fig. 3b). The developed lamellated cristae were found and the dense matrix was filled with the interior of mitochondria from day 26 and day 30 of the hard diet group (Fig. 3c, 3e). The structure of cristae becomes progressively clearer as the time is passed (Fig. 3 g, i, k). On

Fig. 4. Rate of halo area for cross-sectional area of mitochondria in masseter muscle. The halo area was determined by measurement of photograph of mitochondria in fed a hard diet (open circles) or in soft diet (dots). Values are means and standard deviation ($n = 50$, $**P < 0.01$, $*P < 0.05$, Mann-Whitney's U-test).

the other hand, on soft diet group the rate of mitochondria containing halo structure increases. As indicated in Fig. 3h, the area of halo is large and membrane structure of inner space of mitochondria is ambiguous and obvious crista was not observed. The structural ambiguity is recovered on the day 120 (Fig. 3l); that is, the structure of mitochondria on day 120 in soft feeding group is almost the same as those in hard diet group. (Fig. 3k, l).

The percentage of halo area per cross-sectional area of mitochondria is rapidly decreased between day 0 and day 21, and then gradually increased from beginning feeding soft diet until day 60, but that on hard diet group is retained at lower rate than that on soft diet group (Fig. 4). In mitochondria on day 35, the rates of halo area are low value on hard diet group, and the difference on day 35 and 60 between both value of two groups is high, indicating that the mitochondria of soft diet group on day 35 and 60 may be impaired, compared with those of hard diet group. However, the halo rate is similar value in soft and hard diet groups on day 120 days, suggesting that the damage of mitochondria of soft diet group may be recovered.

We then determined the activity of two respiratory chain enzymes contained in the mitochondrial inner membrane (Fig. 5a, b). Both the NADH- O_2 and succinate- O_2 oxidoreductase activities of mitochondria prepared from rats fed the hard diet was higher than that in the soft diet group over most of the period examined after day 20. The differences in NADH- O_2 and succinate- O_2 oxidoreductase activity of mitochondria were largest on day 60 (about 1.8- and 1.4-fold, respectively). The differences in both activities decreased on day 120.

Discussion

In the rat, the masseter muscle is the largest masticatory muscle and it seems to be the most important muscle for biting hard food¹⁴⁾. In this study, we investigated the morphology of the masseter muscle fiber cells and their mitochondria prepared

Fig. 5. Developmental changes in two respiratory chain enzyme activity of isolated mitochondria of rat masseter muscle from rats fed the hard (open circles) or soft diet (dots). a) succinate- O_2 oxidoreductase, b) NADH- O_2 oxidoreductase. Values are means and standard deviation. ($n = 12$, $**P < 0.01$, $*P < 0.05$, Mann-Whitney's U-test)

from rats fed the hard or soft diet after feeding behavior changed from suckling to mastication. Some papers indicated that the size of masseter muscle was significantly smaller in rat kept on a soft diet than those in control rat^{7,8,11,14}). In our results, the fiber cell cross-sectional area and number of mitochondria prepared from rats fed hard diet were greater than those of animals fed soft diet on post-natal day 60, and these differences decreased under both feeding conditions on days 120.

As the cross-sectional area of muscle fiber cells and mitochondrial number per area in the hard diet group were about 1.9- and 1.8-fold greater, respectively, than those in the soft diet group on day 60, the number of mitochondria per cell was calculated as a ratio of about 3.4, suggesting that the function of mitochondrial respiratory chain per one fiber cell unit may be much different between two groups on day 60. This difference, however, decreased on day 120 (the ratio was about 1.6). Muller *et al.* indicated a decrease of the fiber size in rat deep masseter muscle after the insertion of an unilateral occlusal splint, but the plasticity of rodent masseter muscle allows it to adapt within a short period of one week¹⁴). We also observed the plasticity of the rat masseter muscle fiber size and mitochondrial number as mentioned above.

Similar discrepancies were also observed in the enzyme activity of respiratory chain of mitochondrial inner membrane between the two different diet groups. The differences of NADH-O₂ or succinate-O₂ oxidoreductase activity among the both diet group increased on day 60, and decreased on day 120. The activity of oxidases from rats fed the hard diet was higher than that in the soft diet group during the period from day 30 to 120. Succinate-ubiquinone oxidoreductase (complex II) is a mitochondrial marker enzyme that catalyzes oxidation of succinate to fumarate in the tricarboxylic acid cycle and transfers reducing equivalent to mitochondrial membrane-bound ubiquinone^{1,4}). NADH-ubiquinone oxidoreductase (complex I) also transfers electrons from NADH to ubiquinone³). Electrons from both succinate and NADH are sequentially transported via ubiquinone and several common enzymes and finally reduce O₂ by cytochrome c oxidase⁶). Using isolated mitochondria, we measured succinate-O₂ and NADH-O₂ oxidoreductase activity using oxygen electrode, which allowed the monitoring of net electron transfer from both succinate and NADH to O₂. The overall electron transport activity has a close relation to the physiological function of energy supplying system of mitochondria.

We indicated that there was difference between the rate of halo of mitochondria of the hard diet

and that of the soft diet groups, suggesting that the muscle fiber mitochondria from rats fed soft diet was damaged. The muscle damage becomes more extended and severe with time in the soft diet group until day 60, but this damage recovers on day 120, possibly due to an adaptive response of the muscle fiber cells and its mitochondria. Bani *et al.* reported that morphological change of rat masseter muscle fiber cells by occlusal wear, and results showed that occlusal dysfunction leads to morphological damage of muscular fiber and its mitochondria²). They suggested that these changes are likely related to muscle ischemia. Shepard *et al.* studied that the ultrastructure of mitochondria and their cristae in embryonic rat¹⁷). In that paper, the embryo is in a relative stage of hypoxia during early organogenesis and this is associated with decrease of terminal electron transport system activity. In our results we found that good correlation between the time course of damage by soft diet and decrease of respiratory chain activity. On possible explanation to above results is that toxic end-products of decrease of respiratory chain activity namely, hydrogen peroxide, superoxide and hydroxy radicals¹⁷), may result in the increase of halo formation inside the mitochondria. We further showed that the number of mitochondria per cell was about 3.4 fold lower on soft diet group than on hard diet group on day 60 as mentioned above. We also suppose the disappear of mitochondria are caused by the formation of reactive oxygen by the damaged respiratory chain reaction.

In conclusion, we showed that development of masseter muscle fiber cells and their mitochondria is hindered when rats were fed a soft diet, but partially recovered later, and consequently early normal development of masticatory muscle requires eating a hard diet.

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