NADH Dehydrogenase Activity and Expression of mRNA of Complex I (ND1, 51kDa, and 75kDa) in Heart Mitochondria of *klotho* Mouse

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Summary: Mitochondrial enzyme activities and ultrastructure of mitochondria prepared from *klotho* mutant mice were compared with those in wild-type mice. We also measured the levels of expression of ND1, 51kDa, and 75kDa mRNA associated with the genes encoding NADH dehydrogenase and complex I and that of alpha cardiac myosin heavy chain mRNA in both groups. Mitochondrial NADH oxidoreductase activity was higher in *klotho* mutant mice during aging than that in wild-type mice. The area of mitochondria per unit area ($300\ \mu\text{m}^2$) of cell was almost constant from 4 to 7 weeks of age in both groups. A few large mitochondria were scattered between numerous small mitochondria with compact cristae and myofibrils in *klotho* mice from 5 weeks of age. The levels of ND1 and 75kDa mRNA were slightly high from 7 weeks of age in *klotho* mutant mice, whereas they were almost constant in wild-type mice, in spite of reduced expression of alpha cardiac myosin heavy chain mRNA. Our results indicate that *klotho* protein indirectly plays a role in diminished functional adaptability of enzymes in aged heart muscle, and is required for hypertrophy of cardiac mitochondria.

Mice homozygous for the *klotho* mutation (*kl/kl*) are developmentally normal until 3 weeks after birth, but then become inactive and die prematurely at 8 to 9 weeks. The *klotho* mutation is associated with typical age-related phenotypic characteristics, such as a short lifespan, arteriosclerosis, cardiac dysfunction, ectopic calcification, infertility, atrophy of the thymus, and impaired B cell differentiation^{13,16,20–22,25–30}. The *klotho* gene is also generally expressed in tissues associated with calcium homeostasis, such as the kidney, brain, parathyroid gland, lung, and heart¹⁹.

The respiratory complex is a chain of respiratory enzymes related to NADH dehydrogenase and is encoded by both nuclear and mitochondrial genomes. Our previous study revealed differences in the expression of ND1 and 51kDa mRNA in post-natal rat tongue muscle⁸. Increased activity of NADH dehydrogenase is also reportedly associated with structural changes in cristae of mitochondria during development of rat masticatory muscle²³.

The number of mitochondria per cytoplasm of mouse decreased while organelle volume remained constant¹⁰. In contrast, the volume fraction and size of mitochondria decrease, in contrast to an increase of mitochondrial numerical density, in rat heart during aging⁷. Aging-related ultrastructural changes have been found in the heart, such as lipid droplets, lipofuchsin granules, and myelin figures⁷. Large mitochondria are also reported to appear in rat heart under immobilization stress³. Age-dependent changes in mitochondrial structure reflect reduced enzyme activity in heart muscle⁶. However, glucose tolerance, insulin sensitivity and phosphoenolpyruvate carboxykinase (PEPCK) activity in *klotho* mice are higher than those in wild-type mice and the former have less energy expenditure¹⁷. The activities of enzymes involved in the mitochondrial electron transport complexes differ in various tissues during aging¹⁴. Therefore, it is necessary to examine and compare the activity and levels of respiratory enzymes in *klotho* mice

and wild-type mice in terms of the expression of ND1, 51kDa, and 75kDa mRNA associated with the genes encoding NADH dehydrogenase and complex I, in relation to structural changes in the mitochondria of heart muscle, in order to clarify the biological role of *klotho* protein.

Materials and Methods

Transmission electron microscopy

All protocols conformed to the guidelines for the care and used on laboratory animals of Nippon Dental University. The male *klotho* mutant (*kl/kl*) mice and wild-type mice (CLEA JAPAN, INC., Tokyo, Japan; each 4 from 4, 5, 6, 7 weeks olds) were fed a solid pellet diet (hard diet, MF, Oriental Yeast Inc., Tokyo, Japan). Mice were deeply anesthetized with ether and killed by cervical dislocation. Small blocks of cardiac chamber of heart muscle from two groups (*klotho* mutant mice and wild-type mice) were fixed in 2% glutaraldehyde-cacodylate buffer (pH 7.2) for 2 hr at 4°C, and post-fixed 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. Cross and longitudinal thin sections of myofibrils for transmission electron microscopy (TEM) 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. Fifty mitochondria from ten photographs (magnification $\times 10,000$) were selected randomly from each section. The cross-sectional areas of mitochondria per unit area ($300 \mu\text{m}^2$) of cell were measured arbitrarily from several locations using a photo analyzer (Image Gause software, Fuji Film, Tokyo, Japan) linked to a personal computer (EVO 320, CompaQ, Tokyo, Japan). A total of 50 mitochondria in each field were measured.

Isolation of mitochondria

Mitochondria were isolated by the method of Hogeboom (1955) with slight modifications¹¹. The preparation of mitochondria was carried out at 4°C. The samples were stored at -80°C for later use.

Enzyme assays

Mouse heart muscle and liver mitochondria (0.1 g of protein) were used for each assay. NADH- O_2 oxidoreductase activities were 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 NADH (50 mM). Protein concentration was determined by the method of Lowry *et al.* (1951)¹⁵, with bovine serum albumin as a standard.

Analysis of mRNA using RT-PCR

Isolation of total RNA

Immediately following the sacrifice of two groups (*klotho* mutant (*kl/kl*) and wild-type mice), 30 mg heart muscles were used for RT-PCR methods. Samples were scrapped off and stored at -80°C until ready for use. The heart muscle was thawed, cut into small pieces and total RNA was extracted with RNeasy Mini Kit (QIAGEN) as per manufacturer's protocol. Contaminating DNA was removed using an RNase-free DNase set (QIAGEN) and the total RNA was quantified by means of spectrophotometry. The samples were stored at -80°C until further use.

Preparation of probes using gene primers

Using gene primers are shown in Table 1.

Table 1. Oligonucleotide primers used for RT-PCR

Transcript		Sequence	Product (bp)	Gene Bank
ND1	forward	5'-TACGAGCCGTAGCCCAAACA-3'	452	NC_005089
	reverse	5'-GATCGTAACGGAAGCGTGGA-3'		
51kD	forward	5'-GGGCCTACATCTGTGGAGAAGAGA-3'	425	NM_133666
	reverse	5'-CACCAGTGCATCGAAGTCCATTAG-3'		
75kD	forward	5'-CAGGACTTGCCAAAGGATTGTTTC-3'	477	AK036926
	reverse	5'-CTCGGTGACAGCTTTGACACACTT-3'		
MHC-1	forward	5'-GAGCTACTGGAAAGCTGGCTTCTG-3'	490	M76601
	reverse	5'-ACTCTGCCCTTGGTTACATACTC-3'		
GADPH	forward	5'-ACCACAGTCCATGCCATCAC-3'	452	M17701
	reverse	5'-CCACCACCCTGTTGCTGTA-3'		

RT-PCR

The DNA oligonucleotide primers were selected from the nucleotide sequences that have been published for each gene (Table 1). 1 µg of total RNA was reverse transcribed in a 25 µl of the reaction mixture at 42°C for 30 min. This reaction mixture consisted of 0.4 µM oligo (dT)₁₅ primer, 1 mM each dNTP, 20 units of RNase inhibitor (TaKaRa), 17.5 units of AMV reverse transcriptase (TaKaRa), 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM of DTT and 10 mM MgCl₂. For the negative control, a total RNA sample without added reverse transcriptase was tested for DNA contaminants. For PCR, 1 µl of the cDNA mixture was added to 25 µl of master mix. This master mix consisted of 400 µM each dNTP, 0.4 µM each forward primer and reverse primer, 0.5 units of Ampli Taq Gold (Applied Biosystems, Foster, CA). The reactions were performed under different annealing temperature and cycle repetitions: 60°C and 40 cycles for GAPDH; 60°C and 36 cycles for ND1; 60°C and 40 cycles for 51kDa; 60°C and 40 cycles for 75kDa; 60°C and 40 cycles for alpha cardiac myosin heavy chain mRNA (MHC-I). An aliquot of each PCR product was separated on a 1.5% agarose gel and stained with ethidium bromide. The stained gels were photographed under ultraviolet illuminator using Polaroid film. The intensity of the bands was quantified using Image Gause software (Fuji Film, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by employing commercially available software (Microsoft® office Excel 2003). Differences between hearts muscle in *klotho* mutant (*kl/kl*) and wild-type mice were tested using the t-test. Correlations between groups of values were evaluated calculating the best fit, based on least-squares regression analysis. The regression lines were then represented and the coefficient of correlation (R) was indicated. For the statistical analyses, significance was accepted at $p < 0.05$ (*¹, show in figures) and $p < 0.01$ (*², show in figures), and $p < 0.001$ (*³, show in figures).

Results

The body weight of *klotho* mutant mice was almost constant from 4 to 7 weeks, and was lower than that of wild-type mice (Fig. 1). We determined the activities of one respiratory chain enzyme contained in the mitochondrial inner membrane (Fig. 2). In *klotho* mutant mice, NADH-O₂ oxidoreductase activity of the mitochondrial respiratory

Fig. 1. Changes of body weight of the *klotho* mutant mice (KT) and wild-type mice (WT) during development.

chain in heart muscle increased rapidly from 6 weeks of age (Fig. 2a), whereas the levels of other mitochondrial proteins decreased from the same time point (total protein/mg: 5 weeks, 147.5 mg/ml per 1 g; 6 weeks, 19.0 mg/ml per 1 g; 7 weeks, 6.1 mg/ml per 1 g), and were lower than those of wild-type mice (total protein/mg: 5 weeks, 205.3 mg/ml per 1 g; 6 weeks, 110.2 mg/ml per 1 g; 7 weeks, 47.1 mg/ml per 1 g). NADH-O₂ oxidoreductase activity of the mitochondrial respiratory chain in the liver decreased gradually from 5 weeks onwards in *klotho* mutant mice (total protein/mg: 5 weeks, 15.6 mg/ml per 1 g; 6 weeks, 105.3 mg/ml per 1 g; 7 weeks, 99.6 mg/ml per 1 g), and was drastically lower than that in 6- and 7-week-old wild-type mice (total protein/mg: 5 weeks, 16.4 mg/ml per 1 g; 6 weeks, 717.3 mg/ml per 1 g; 7 weeks, 448.1 mg/ml per 1 g) (Fig. 2b).

The size of mitochondrion heart muscle was almost constant throughout the study period (Fig. 3). There were no differences in the size of mitochondrion between *klotho* mutants and wild-type mice. We also examined differences in other mitochondrial features. In the mutants, loss of cristae membranes occurred mainly in large mitochondria, which gradually increased in number and showed a scattered distribution in myocardial cells (Fig. 4).

We also measured the levels of expression of ND1, 51kDa mRNA, and 75kDa associated with the genes encoding NADH dehydrogenase and complex I (Fig. 5a). In *klotho* mutant mice, the expression of ND1 and 75kDa mRNA was constant

Fig. 2. Developmental changes in respiratory chain enzyme activity (NADH-O₂ oxidoreductase) of isolated mitochondria of heart muscle (a) and liver (b) from the *klotho* mutant mice (KT) and wild-type mice (WT). Values are means and standard deviation. $p < 0.01$ (*²), $p < 0.001$ (*³).

from 5 to 6 weeks of age, and slightly higher at 7 weeks of age than that in wild-type mice, which was almost constant for 4 weeks (Figs. 5b, d). The expression level of 51kDa mRNA was almost constant in both *klotho* mutant and wild-type mice (Fig. 5c). In contrast, the expression level of alpha cardiac myosin heavy chain mRNA increased gradually from 5 weeks of age in both groups, and was lower at 7 weeks of age in *klotho* mutant mice than in wild-type mice (Figs. 6a, b).

Fig. 3. Developmental changes in measurement (cross-sectional area of mitochondria) of heart mitochondria of the *klotho* mutant mice (KT) and wild-type mice (WT). Values are means and standard deviation. $p < 0.001$ (*³).

Discussion

It has been reported that there is a decline in total aerobic NADH oxidation (coenzyme Q reductase and cytochrome oxidase activities), but no change in ubiquinol-cytochrome *c* reductase activity in hearts of rats aged 24 months¹⁰. It is expected that in the type of aging shown by *klotho* mutant mice, the energy-producing system runs at a low basic level because of atrophy of the pancreas. The *klotho* mutant mouse possesses less stored energy in the form of glycogen in the liver¹⁷. Our present study showed that the enzyme activity of the respiratory chain in heart mitochondria of *klotho* mutant mice increased rapidly during aging, and that the levels were higher than in wild-type mice at each stage. In contrast, respiratory chain enzyme activity in liver mitochondria of *klotho* mutant mice decreased during aging, although the level of activity was higher than that of wild-type mice from 4 to 7 weeks of age. In rat heart mitochondria, NADH cytochrome *c* reductase and cytochrome oxidase activities are known to increase up to 12 months of age, and then decrease at 18–26 months⁵. However, in the present study, total mitochondrial proteins isolated from heart muscle of *klotho* mutant mice were decreased from 6 weeks of age, and the levels of all of them were lower than those of wild-type mice throughout the

Fig. 4. Electron micrographs of heart mitochondria (mt) in the *klotho* mutant mice (b, d, f, h) and wild-type mice (a, c, e, g). A few large mitochondria with disappeared cristae (arrows) around the membranes and the swelling and vacuolation of mitochondria partial found in cells. a, b) 4 weeks of age, c, d) 5 weeks of age, e, f) 6 weeks of age, g, h) 7 weeks of age, (a–c, e, g, h: bar = 1 μm; d, f: bar = 400 nm).

Fig. 5. Semi-quantification of complex I (ND1, 51kDa, 75kDa) and (GAPDH) mRNA by RT-PCR in heart mitochondria of the *klotho* mutant mice (KT) and wild-type mice (WT). Gel-electrophoretic pattern of ND1, 51kDa, 75kDa and GAPDH (a) and relative changes in ND1 (b), 51kDa (c), and 75kDa (d) mRNA of heart mitochondria on 4-, 5-, 6-, and 7 weeks of ages. Values are means and standard deviation. $p < 0.05(*^1)$, $p < 0.01(*^2)$

Fig. 6. Semi-quantification of cardiac myosin heavy chain (MHC-1) and GAPDH mRNA by RT-PCR in heart mitochondria of the *klotho* mutant mice (KT) and wild-type mice (WT). Gel-electrophoretic pattern of MHC-1 and GAPDH (a) and relative changes in MHC-1 (b) mRNA of heart mitochondria on 4-, 5-, 6-, and 7 weeks of ages.

study period. Our findings suggested that in *klotho* mutant mice the potential for mitochondrial enzyme activity in the heart is high during aging, in spite of the reduced amount of mitochondrial protein. Our previous study showed that muscle mitochondrial enzyme activity was higher during hibernation than during active life²⁴⁾. Similar results have been reported for some species of fish^{1,18)}. Activities of some individual complexes are known to be decreased in old animals, but no common pattern can be discerned among various tissues¹⁴⁾. Stored glycogen is slowly utilized to produce energy in response to aging or stress, and in *klotho* mutant mice compared with wild-type mice, the respiratory chain in heart mitochondria functions actively, in contrast to liver mitochondria.

51kDa and 75kDa mRNA from the nuclear genome and ND1 mRNA from the mitochondrial genome encode key protein subunits for the formation of NADH dehydrogenase^{2,8)}. Our study showed that the levels of ND1 and 75kDa mRNA

were slightly higher in *klotho* mutant mice from 7 weeks of age, whereas they remained almost constant in wild-type mice. These findings suggest that the high level of enzyme activity in *klotho* mutant mice at 3–6 weeks of age is not due to enzyme regulation. As heart muscle is unable to receive sufficient glucose from the blood¹⁷⁾, then the high enzyme activity in *klotho* mutants might serve to maintain normal circulatory conditions, except in 7-week-old mice. Hypertension can cause a reduction in the level of mRNA for cardiac sarcoplasmic reticular calcium-activated ATPase⁴⁾. However, the level of alpha-skeletal actin mRNA is lower in aged skeletal muscle and uterus, but higher in aged myocardium¹²⁾. Therefore, the high expression of ND1 and 75kDa mRNA in the mitochondria at 7 weeks of age in *klotho* mutant mice is possibly due to gene derepression caused by hypertrophy of cardiac mitochondria and myocytes.

In this study, we investigated the ultrastructure of the mitochondria prepared from *klotho* mutant and wild-type mice. TEM indicated that large mitochondria showing loss of cristae were present in the *klotho* mutant mice from 5 to 7 weeks of age. Our results also indicated reduced levels of alpha cardiac myosin heavy chain mRNA from 7 weeks of age. Buttrick *et al.* (1991) reported a 46% reduction in alpha myosin heavy chain mRNA in 24-month-old rats in comparison with 4-month-old rats⁴⁾. Frenzel and Feiman (1984) also observed a reduction in the transverse diameter of ventricular myofibrils in aged (2-year-old) rats⁷⁾. Therefore the functional adaptability of heart myofibrils is diminished in aged rats in comparison with *klotho* mutant mice.

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