Expression of respiratory chain enzyme mRNA and the morphological properties of mitochondria in the masseter muscles of klotho mutant mice

By

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Summary: The activity of respiratory chain enzymes in a rat’s masseter muscle changes as the animal ages; however, there is little information about the RNA transcript levels of mitochondrial enzymes in klotho mutant mice as they age. We measured the activities of NADH-ferricyanide oxidoreductase and NADH-O2 oxidoreductase, and the RNA transcript levels of NADH dehydrogenase, the mitochondrial isoform of ND1, the nuclear isoforms of the 51 kDa and 75 kDa subunits of Complex I, the nuclear isoform of cytochrome c, and the mitochondrial isoform of beta subunits of ATPase (Complex V). In addition, we measured the RNA transcript levels of catalase (CAT) and superoxide dismutase (SOD), which are associated with antioxidant proteins. Moreover, we measured ATP concentrations using a luciferin-luciferase assay, and we determined the amount of cytochrome c associated with mitochondria in both klotho mutant mice and wild-type mice. However, the mRNA levels of cytochrome c and Complex V components, the mRNA levels of CAT, SOD, and apoptosis-inducing factor (Aifm), and the protein level of cytochrome c remained constant as klotho mutant mice aged from 5 weeks to 7 weeks. In wild-type mice, these components (except for those of Complex I) increased over time. NADH-ferricyanide oxidoreductase and NADH-O2 oxidoreductase activities decreased in klotho mutant mice as they aged from 5 weeks to 7 weeks. A few large mitochondria were scattered between myofibrils, and 7-week-old klotho mutant mice displayed an increased number of irregular mitochondria with fewer cristae. Our results indicate that the klotho protein plays a role in the diminished functional adaptability of enzymes in the masseter muscle of klotho mutant mice throughout the aging process.

Introduction

The aerobic electron transport chain is composed of four protein complexes located in the inner membrane of mitochondria. Complex I (also known as NADH-ubiquinone oxidoreductase) transfers electrons derived from the oxidation of reduced nicotinamide adenine dinucleotide (NADH), and Complex II (also known as succinate-ubiquinone oxidoreductase) transfers electrons derived from the oxidation of succinate. Complexes I, III (ubiquinol-cytochrome c oxidoreductase), and IV (cytochrome c oxidase) are proton exchangers that maintain the membrane potential. These complexes transfer electrons to a subsequent complex or to molecular oxygen and transfer protons out of the mitochondrial matrix. The final complex of the oxidative phosphorylation machinery, Complex V (ATP synthase (F0F1 complex)), allows protons to flow back into the mitochondrial matrix through its F0 subunit and uses the energy from the proton gradient to drive the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) in the F1 subunit (Stock et al., 1999; Gao et al., 2005; van Raam et al., 2008). NADH-ubiquinone oxidoreductase (Complex I) is composed of many subunits, 34 of which are encoded by the nuclear genome, and 7 of which are encoded by the mitochondrial genome. The ND1 subunit is the binding site for quinone, the electron acceptor of complex I in the electron transport chain (Hatefi, 1985; Robinson, 1998; Schapira, 1998; Genova et al., 2004), and the 51 kDa and 75 kDa subunits are involved in the transfer of electrons to NADH (Brandt, 1997; Robinson, 1998). Our previous study revealed differences in the expression of ND1 and in the mRNA level of the 51 kDa protein in the tongue muscle of postnatal rats (Fujita and Sato, 2003). The activities of the enzymes involved in the mitochondrial electron transport differ in various tissues.

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During aging (Kwong and Sohal, 2000). Additionally, the concentrations of mitochondria-encoded mRNAs found in skeletal muscle also decrease as an animal ages (Barazzoni et al., 2000; Welle et al., 2000, 2003). In contrast, the masseter muscle displays specific profiles for the various enzymes (cytochromes b, c, c1, a, and a3; ATP synthase; and succinate and NADH dehydrogenases) (Guerinckx et al., 1986; Kiliaridis et al., 1988; Easton and Carlson, 1990; Miyata et al., 1993; Miehe et al., 1999; Nishide et al., 2001). In rat heart muscle, the activities of NADH-dehydrogenase and the ATP synthase are reduced, as is the capacity for oxygen utilization and ATP synthesis (Preston et al., 2008); however, there is no reduction in mitochondrial integral membrane protein during aging (Navarro and Boveris, 2007) and no change in ATPase activity in the liver, heart, or skeletal muscle (Barogi et al., 1995). No difference has been found in the distribution of intermediate ATPase-stained fibers between the masseter muscles from young and very old humans; this suggests that aging affects various human muscles in different ways (Kirkby and Garbarsch, 2000). In the tissues of some species, a decline in the respiratory chain capacity is seen with aging, and cytochrome c oxidase-deficient (COX(-)) muscle fiber is related to the mitochondrial content (Muller-Hocker, 1992). The COX(-) fibers reduced mitochondrial content by enhanced contractile activity (Skorjanc et al., 2001). These reports indicate that various tissues exhibit different metabolism profiles. The levels of enzyme activity in rat masseter muscles change with fasting and re-feeding in addition to aging (Nortone et al., 2001; Sato and Konishi, 2004). Moreover, increased NADH dehydrogenase activity is also associated with structural changes in the cristae of mitochondria during the development of rat masticatory muscles (Sato et al., 1998). However, it is not known how mitochondrial properties and the respiratory enzymes associated with the energy-producing system are correlated in the masseter muscle during aging. The activities of mitochondrial free radical scavengers such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase differ significantly with age in skeletal muscle (Capel et al., 2004); these differences are unrelated to the mitochondrial oxidative capacity of skeletal muscle (Servais et al., 2003). The activities of the enzymes involved in the mitochondrial electron transport complexes differ in various tissues during aging (Kwong and Sohal, 2000). The levels of enzyme activity in the rat masseter muscle were found to change throughout the aging process (Norton et al., 2001). A few reports have investigated the relationship between the mRNA levels of mitochondrial enzymes, such as respiratory chain enzymes and aging. While the masseter muscle is involved in mastication, speaking, and swallowing, skeletal muscle plays a more structural role.

The klotho mutant mouse displays typical age-related characteristics at an earlier age than wild-type mice (Kuro-o et al., 1997; Saito et al., 1998; Yamashita et al., 1998; Okada et al., 2000; Saito et al., 2000; Suga et al., 2000; Utsugi et al., 2000; Yamashita et al., 2000a, b; Manabe et al., 2001; Yamashita et al., 2001). The amount of klotho protein declines in various tissues during aging (Nabeshima, 2002; Shih and Yen, 2007), and the klotho gene is generally expressed in tissues associated with calcium homeostasis, such as the kidney and heart (Nabeshima, 2002). The calcium concentration is related to muscular enzyme activity. Glucose tolerance, insulin sensitivity, and phosphoenolpyruvate carboxykinase (PEPCK) activity are different in klotho mutant mice and wild-type mice, and the former exhibit lower energy expenditure (Mori et al., 2000). However, it is not yet known how the klotho protein in the masseter muscle is related to aging. Therefore, it is necessary to examine the levels of respiratory enzymes in the masseter muscles of klotho mutant mice as they age. In the present study, we examined the activities of NADH-ferricyanide oxidoreductase and NADH-O2 oxidoreductase and the mRNA expression of NADH dehydrogenase (ND1, 51 kDa, and 75 kDa), cytochrome c, and beta subunits of ATP synthase. We also assessed the levels of oxidative stress and the morphological properties of mitochondria in masseter muscles from 5–7-week-old klotho mutant and wild-type mice.

Materials and Methods

Animals

All laboratory animals were procured from the Nippon Medical Science Animal Resource Laboratory and were bred at the Animal Testing Center of the Department of Dentistry at Nippon Dental University. Male klotho mutant mice and wild-type mice (CLEA JAPN. INC, Tokyo, Japan) were fed a solid pellet diet (MF, Oriental Yeast Inc., Tokyo, Japan), beginning at 4 weeks of age. The animals were sacrificed by an overdose of pentobarbital, and their masseter muscles were removed. Fresh samples of right masseter muscle were prepared from each klotho mutant (N = 24) and wild-type mouse (N = 24), and specimens from these animals at each stage were analyzed. Real-time RT-PCR was used to measure mRNA levels (n = 4), and protein levels were examined by western blot analysis (n = 4). Light and electron microscopy studies (n = 4) were also completed.

Ethics

The study followed the regulations of Nippon Dental University (i.e., Rules for the Care and Use of Laboratory Animals, no. 03–34).

Transmission electron microscopy

Four samples from each of the two groups (klotho mutant and wild-type mice) at 5 weeks and 7 weeks of
Mitochondria in the masseter muscle of klotho mutant mice

Age were fixed in 2% glutaraldehyde-cacodylate buffer (pH 7.2) for 2 hr at 4°C; they were post-fixed in 1% osmic acid for 1 hr at 4°C. After they were washed with cacodylate buffer (pH 7.2), the samples were dehydrated in absolute ethyl alcohol and then embedded in Epon 812. Thin sections of myofibrils 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 (TEM) operating at 80 kV was used for the observations. Fifty mitochondria from ten photographs (magnification ×10,000) were selected randomly from each section between klotho mutant and wild-type mice. The number of mitochondrial cristae loss (halo) in masseter muscle was measured arbitrarily from several locations.

Isolation of Mitochondria

Mitochondria were isolated according to a modified version of the method described by Hogeboom (1955). The preparation of mitochondria was carried out at 4°C, and the samples were stored at −70°C for later use.

Enzyme assays

Mouse masseter muscle mitochondria (0.1 g of protein) were used for each assay. NADH-ferricyanide oxidoreductase activity was determined by monitoring the decrease in absorbance at 440 nm associated with the reduction of ferricyanide in 3-ml reaction mixture containing 50 mM Tris-HCl (pH 7.4), 2 mM KCN, and 1 mM K₃Fe(CN)₆. NADH-O₂ oxidoreductase activity was measured polarographically using a Clark-type oxygen electrode (Yellow Spring Ltd., Ohio). The oxidase activity assay was performed with 50 mM Tris-HCl (pH 7.5) at 25°C in a closed 2 ml reaction chamber equipped with a magnetic stirrer; the rate of oxygen uptake was monitored. Each reaction was initiated by the addition of substrate NADH (500 µM). The protein concentration was determined according to the method described by Lowry et al. (1951), using bovine serum albumin as a standard.

Analysis of mRNA by real-time RT-PCR

Isolation of total RNA

Immediately after sacrifice, the masseter muscles were removed from each mouse by scraping and were stored at −80°C. After the muscles were cut into small pieces, the samples (0.1 g) were used for RNA isolation. Total RNA was extracted with a Quick Prep Total RNA Extraction Kit (Amersham Biosciences, UK) according to the manufacturer’s protocol. Contaminating DNA was removed using an RNase-free DNase (DNA-free, Ambion, Austin, Texas USA), and total RNA was quantified by spectrophotometry. The samples were stored at −80°C until further use.

RT-PCR

DNA oligonucleotide primers were selected based on the published nucleotide sequences of each gene (Table 1). A sample of total RNA (1 µg) was reverse transcribed in a 25-µl reaction mixture at 50°C for 1 hr. This reaction mixture consisted of 0.4 µM oligo (dT)15 primer, 1 mM of each dNTP, 20 units of RNase inhibitor (TaKaRa), 2.5 units of AMV reverse transcriptase (TaKaRa), 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM DTT, and 5 mM MgCl₂. For the negative control, a reaction mixture was prepared without reverse transcriptase and was tested for DNA contaminants. For the PCR, 1 µl of the cDNA mixture was added to 25 µl of master mix. The master mix consisted of 200 µM of each dNTP, 0.4 µM of forward primer, 0.4 µM of reverse primer, and 0.625 units of Ampli Taq Gold (Applied Biosystems, Foster, CA).

Table 1 Oligonucleotide primers used for RT-PCR

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<td>beta subunits of ATP synthase</td>
<td>TGCAGGCGGCGCCCAAGAAAATGACGAGCTGCCAAGACACAA-3'</td>
<td>NM_016774.2</td>
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<tr>
<td>cytochrome C</td>
<td>CATGCGGCTGATGACGAGCTGCCAAGACACAA-3'</td>
<td>NM_025899.2</td>
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<tr>
<td>catalase</td>
<td>GGGCCCTACACTTGCTGGAAGAAGAGA-3'</td>
<td>NM_098804.1</td>
</tr>
<tr>
<td>myosin heavy chain IIa</td>
<td>AGCTGCTGATGACGAGCTGCCAAGACACAA-3'</td>
<td>NM_144961</td>
</tr>
<tr>
<td>ND1</td>
<td>TGCAGGCGGCGCCCAAGAAAATGACGAGCTGCCAAGACACAA-3'</td>
<td>NM_133666</td>
</tr>
<tr>
<td>51KD</td>
<td>GATCGGCTGATGACGAGCTGCCAAGACACAA-3'</td>
<td>AK036926</td>
</tr>
<tr>
<td>75KD</td>
<td>GGGCCCTACACTTGCTGGAAGAAGAGA-3'</td>
<td>NC050089</td>
</tr>
<tr>
<td>GADPH</td>
<td>ACCACAGTGCACGCTGCCATCAC-3'</td>
<td>M17701</td>
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<table>
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<td>452</td>
<td>NM_133666</td>
</tr>
<tr>
<td>51KD</td>
<td>5'-GATCGGCTGATGACGAGCTGCCAAGACACAA-3'</td>
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<tr>
<td>75KD</td>
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<td>GADPH</td>
<td>5'-ACCACAGTGCACGCTGCCATCAC-3'</td>
<td>452</td>
<td>M17701</td>
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</table>
The reactions were performed using different annealing temperatures and different numbers of cycles: 60°C and 28 cycles for GAPDH; 60°C and 40 cycles for ND1; 55°C and 40 cycles for 51 kDa; and 60°C and 40 cycles for 75 kDa. An aliquot of each PCR product was separated on a 1.5% agarose gel and then stained with ethidium bromide. The stained gels were photographed under an ultraviolet illuminator using Polaroid film. The intensity of the bands was quantified using Image Gause software (Fuji Film, Tokyo, Japan).

**Quantitative real-time RT-PCR**

Quantitative real-time RT-PCR was performed using an Applied Biosystems 7300 Fast Real-Time PCR System, following the protocol recommended by the manufacturer. Each amplification mixture (50 µl) contained 100 ng of cDNA, 900 nM of forward primer, 900 nM of reverse primer, 250 nM fluorogenic probe, and 25 µl of Universal PCR Master Mix (Applied Biosystems). The cycling parameters for the PCR were 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min with beta subunits of ATP synthase (Atp5β: Applied Biosystems, Mm00443967_g1), cytochrome c (Uqcr2: Applied Biosystems, Mm00445959_m1), catalase (CAT: Applied Biosystems, Mm00437992_m1), and apoptosis-inducing factor associated with mitochondria (Aif1: Applied Biosystems, Mm00442540_m1). The levels of the amplified mouse cDNAs were normalized to that of GAPDH (rodent GAPDH primers and probes were obtained from Applied Biosystems, ‘Assays-On-Demand’). The threshold cycle (Ct), defined as the cycle at which the amplification of the PCR product enters the exponential phase, was determined for each gene by plotting the fluorescence level versus the cycle number on a logarithmic scale. The relative expression levels of the genes (Atp5β, Uqcr2, and CAT) were estimated by calculating the ΔCt value, defined as the difference in the Ct values of the targets and the reference gene (GAPDH), as recommended by the supplier. The ΔCt was inversely proportional to the level of each mRNA transcript present in the muscle samples of the mice; a high ΔCt value corresponded to a lower mRNA level.

**Immunoblot analysis**

Mitochondria were isolated according to the method described by Hogeboom [1955]. To prepare the myosin fractions, the mouse masseter muscles were homogenized in cold STE solution (0.25 M sucrose, 1 mM Tris-HCl (pH 7.4), 0.1 mM EDTA). The supernatant was centrifuged at 5,500 x g for 20 min at 4°C. The second precipitate was re-suspended in 10 ml of STE solution and re-centrifuged twice at 6,000 x g for 15 min. The presence of the mitochondria-specific marker cytochrome c was assessed by western blot analysis (cytochrome c, sc-13156, Santa Cruz Biotech. INC, CA, USA) to confirm that mitochondria were found in the mitochondrial fraction.

**Luciferin-luciferase reaction**

After isolation, the mitochondria were repeatedly washed by perfusion with the original suspension medium. In the assay, 100 µl of mixture buffer was mixed with 50 µl of the sample (LL-100-IKinshirou ATP Assay System, TOYO B-Net CO., LTD, Tokyo, Japan). The protein volume was calculated using the BioRad protein assay. The calibrations for the assay were carried out in 10 mM Hepes (pH 7.45). The concentration of ATP was measured using a conventional procedure with a GloMax®-Multi Detection System (Promega, Wisconsin, U.S.A.).

**Antioxidant Activity**

The assay was carried out using a superoxide anion-2-methyl-6-methoxyphenylethynylimidazopyrazynone (MPEC) reaction kit (ATTO Corp. Osaka, Japan) according to the manufacturer’s instructions. Light emission induced by xanthine oxidase (XO) is inhibited by XO inhibitor or a radical scavenger. We measured the generation of products by XO, such as O$_2^-$ and H$_2$O$_2$, as a deleterious effect on lifespan using the chemiluminescent probes MPEC and DCFH-DA. MPEC and DCFH-DA are primarily sensitive to O$_2^-$ and H$_2$O$_2$, respectively (ATTO, Japan). Light emission was measured with a GloMax®-Multi Detection System (Promega, Wisconsin, U.S.A.).

**Statistical analyses**

Data are presented as means ± SEM. Differences between two groups with respect to mean analysis data was assessed with a one-way analysis of variance (ANOVA). A Student’s t-test was used to test the statistical differences among values obtained from these data. Correlations between groups of values were evaluated by calculating the best-fit line using a least-squares regression analysis. The regression lines and the correlation coefficients (R) were shown in Fig. For the statistical analyses, significance was accepted at three levels: p < 0.05 (*), shown in figures), p < 0.01 (**, shown in figures), and p < 0.001 (***, shown in figures).

**Results**

**Expression of the klotho protein in klotho mutant and wild-type mice between 5 weeks and 7 weeks of age.**

The klotho mRNA was detected in all of the masseter muscles from wild-type mice, whereas no klotho protein was detected in the masseter muscles from klotho mutant mice (Fig. 1a). The levels of this mRNA increased as the wild-type mice aged from 4 weeks to 6 weeks, and the expression dropped as the mice aged at 7 weeks of age (Fig. 1b).
Mitochondria in the masseter muscle of klotho mutant mice

NADH-ferricyanide oxidoreductase activity and NADH-O₂ oxidoreductase activity in 5−7-week-old klotho mutant and wild-type mice.

The NADH-ferricyanide oxidoreductase activity of the mitochondrial respiratory chain in the masseter muscle of klotho mutant mice was low at 7 weeks of age (p < 0.05); in contrast, in the wild-type mice, the reductase activity was almost constant between 5 weeks and 7 weeks of age (Fig. 2a). The NADH-O₂ oxidoreductase activity in the masseter muscles of klotho mutant mice was low at 7 weeks of age, while the level of this enzyme was high in 5−7-week-old wild-type mice (Fig. 2b).

Amount of ATP and SOD between 5 and 7 weeks of age in klotho mutant and wild-type mice.

Figure 3 shows the ATP concentrations and SOD volumes of the mice. The levels of ATP in wild-type mice were higher than those in the klotho mutant mice at 7 weeks of age (Fig. 3a). In contrast, the level of SOD was very high in the klotho mutant mice at 7 weeks of age, but was only moderately high in the wild-type mice between 5 weeks and 7 weeks of age (Fig. 3b).

Structure of mitochondria between 5 weeks and 7 weeks of age in klotho mutant and wild-type mice.

In the klotho mutant mice, the loss of cristae membranes occurred mainly in the large mitochondria, and
these mitochondria displayed a scattered distribution in masseter muscle (Figs. 4a–d). The percentage of irregular, large mitochondria gradually increased during the aging process and was elevated in 7-week-old klotho mutant mice (Fig. 5).

Expression of ND1, 51 kDa and 75 kDa, and MyHC mRNAs in klotho mutant and wild-type mice between 5 and 7 weeks of age.

The expression pattern of mRNAs for Complex I components (ND1, 51 kDa, and 75 kDa) is shown in Figs. 6a–d. The expression pattern of mRNAs for ND1 and 51 kDa in the klotho mutant mice was similar to the low pattern of expression observed in wild-type mice during aging. The mRNA levels of 51 kDa were slightly lower in 7-week-old klotho mutant mice than in wild-type mice (Figs. 6a–c).

Expression of mRNAs for cytochrome c, beta subunits of ATP synthase, catalase, and apoptosis-inducing factor associated with mitochondria in 5–7-week-old klotho mutant and wild-type mice.

The expression patterns of mRNAs for cytochrome c, beta subunits of ATP synthase, and catalase are shown in Fig. 7. The expression pattern of mRNAs for myosin heavy chain IIa was similar in the klotho mutant and wild-type mice from 5–7 weeks of age. The level of mRNAs for cytochrome c, beta subunits of ATP synthase, and catalase were higher in the muscles of 7-week-old wild-type mice than in the muscles of 5-week-old wild-type mice. However, the expression levels of these mRNAs remained almost constant between 5-week-old and 7-week-old klotho mutant mice (Figs. 7a–e).

Immunocytochemistry of cytochrome c in 4–7-week-old klotho mutant and wild-type mice.

Western blot analysis was used to assess the level of the cytochrome c protein in the masseter muscle of 5–7-week-old klotho mutant and wild-type mice. Cytochrome c expression remained almost constant in the masseter muscle of the klotho mutant mice but increased in the wild-type mice at 7 weeks of age (Fig. 8).
Mitochondria in the masseter muscle of klotho mutant mice

Discussion

Previous studies have examined the role of the klotho protein in tissues associated with calcium homeostasis, such as the kidneys, but these studies have not investigated the expression of the klotho protein in skeletal muscle during aging (Kuro-o et al., 1997; Saito et al., 1998; Yamashita et al., 1998; Okada et al., 2000; Saito et al., 2000; Suga et al., 2000; Utsugi et al., 2000; Yamashita et al., 2000a, b; Manabe et al., 2001; Yamashita et al., 2001). Researchers may have assumed that klotho does not play an important role in the regulation of the respiratory chain enzymes in skeletal muscle during aging.

However, our results reveal that the klotho protein was detected in the masseter muscle of wild-type mice and was not detected in klotho mutant mice. We also showed that the levels of this protein increased as the wild-type mice aged from 4 to 6 weeks and decreased when they reached 7 weeks. Nabeshima (2002) reported that the klotho protein decreased in various other mouse tissues during aging, and Shih and Yen (2007) reported that klotho expression declined in rat livers over time. Therefore, klotho protein expression may also be lower in the masseter muscles of older mice. The function of the klotho protein in the enzyme activity of the respiratory chain of mitochondria in the masseter muscle during aging remains unknown. Here, we aimed to define the relationship between masseter muscle klotho protein expression and the expression of RNA transcripts of several enzymes of the mitochondrial respiratory chain. Several reports have indicated that different levels of respiratory chain enzymes associated with aging. In one study, researchers observed a decline in total aerobic NADH oxidation (coenzyme Q reductase and cytochrome oxidase activities) but no change in ubiquinol-cytochrome c reductase activity in the mitochondria of hearts from 24-month-old rats (Herbener, 1976). In another study, the relative concentrations of mtDNA and a representative mtDNA transcript encoding cytochrome c oxidase were found to be lower in the skeletal muscle of older human subjects (Welle et al., 2003). In the klotho mutant mouse, the energy-producing system runs at a low basal level due to atrophy of the pancreas. The klotho mutant mouse also stores less energy in the form of glycogen in the liver (Mori et al., 2000). The present study showed that the enzyme activity of the respiratory chain in the mitochondria of the masseter muscle from klotho mutant mice also decreased at 7 weeks of age, and it suggests that the mitochondria synthesis associated with energy-producing enzyme may be gradually declined in klotho mutant mice. In our results, the mRNA expression of the ND1 and 75 kDa subunits remained almost constant in the klotho mutant mice as they aged from 5 to 7 weeks; in the wild-type mice, the expression of these subunits was slightly decreased at 7 weeks of age. However, the mRNA levels of 51 kDa were reduced in 7-week-old klotho mutant mice. The enzyme activities of both NADH-ferricyanide oxidoreductase and NADH-O_2 oxidoreductase in the masseter muscle of klotho mutant mice were also lower than they were in the 7-week-old wild-type mice. The constant level of NADH-ferricyanide oxidoreductase activity and the slightly increased level of NADH-O_2 oxidoreductase activity in the wild-type mice indicate that the klotho protein is related to the mitochondria synthesis associated with NADH enzyme; this level of synthesis is slightly reduced in klotho mutant mice at 7 weeks of age. The nuclear genome-encoded 51 kDa subunit may be slightly more affected by the klotho protein during aging than the ND1 and 75 kDa subunits are. Reduced ATPase activity occurs during aging, and both the F_0 and F_1 contents of the mitochondria in rat livers vary with age (Guerrieri et al., 1992). Bakala et al. (2003)
indicated that there is a decrease in ATP-stimulated matrix proteolytic activity in rat liver mitochondria isolated from 27-month-old animals. The abundance of mtDNA and mRNA and mitochondrial ATP production all decline with advancing age in human skeletal muscle (Short et al., 2005). Mitochondrial ATPase is one of the important elements of the respiratory chain enzyme; however, levels of ATP differ during aging (Navarro and Boveris, 2007; Barogi et al., 1995). The ATP synthase complex of mitochondria is a multiprotein complex of the inner membrane composed of two oligomeric moieties, $F_0$ and $F_1$. Of the 13 polypeptides of the mammalian ATP synthase, 11 are encoded by nuclear genes and 2 are encoded by mitochondrial genes (Guerrieri et al., 1993). The ATP synthase makes use of the potential energy of the proton gradient produced by the electron transport chain complexes, including cytochrome $c$ oxidase, to synthesize ATP from ADP and Pi (Senior, 1988). The hydrophilic
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F₁ component contains catalytic sites for ATP synthesis, and the F₉, which is embedded in the inner mitochondrial membrane, contains a proton channel (Senior, 1988). F₁ is further composed of α, β, γ, δ, and ε subunits, whereas F₉ is composed of a, b, c (Abrahams et al., 1994; Leyva et al., 2003). The beta subunits of ATP synthase are also composed of one functional unit for ATP synthesis. In the present study, the mRNA levels of beta subunits of ATP synthase isolated from the masseter muscles of klotho mutant mice decreased as the mice aged from 5 weeks to 7 weeks whereas the expression in wild-type mice increased as they aged. These results suggest that the klotho protein affects ATP synthesis in the mitochondria of the mouse masseter muscle.

The mammalian cytochrome c oxidase enzyme complex consists of 13 subunits; 3 subunits are encoded by mitochondrial genes, and the remaining 10 subunits are encoded by nuclear genes (Wallace, 1992). Cytochrome c release is related to mitochondrial dysfunction, including oxidative damage and apoptosis, in the brains of aging C57BL6 mice (Manczak et al., 2005). Reductions in the mtDNA copy number (−23–40%) and in COX activity (−32%) occurred in skeletal muscle groups (Barazzoni et al., 2000). The level of mRNA for mitochondrial enzymes (cytochrome c) in the masseter muscles of klotho mutant mice also declined as the mice age from 5 to 7 weeks; in contrast, the mRNA for mitochondrial enzymes increased in the wild-type mice during this same period.

Thus, cytochrome c is an aging protein in masseter muscle, and functional klotho protein also affects cytochrome c synthesis in masseter muscle during aging. Significant age-related increases in the activities of manganese superoxide dismutase (Mn-SOD), selenium-dependent glutathione peroxidase (GPX), and catalase (CAT) have been detected in the interfibrillar mitochondria from the heart muscle (Judge et al., 2005). CAT is a marker of oxidative stress. The level of CAT mRNA remained almost constant as klotho mutant mice aged from 5 to 7 weeks, but it increased in wild-type mice during aging. Thus, oxidative damage occurs as early as 5 weeks of age in klotho mutant mice, and this damage may result in low levels of activity in respiratory chain enzymes. Therefore, we determined that the accumulation of oxidant-induced damage occurred in the masseter muscle of klotho mutant mice and that this damage contributed to age-related alterations in muscle function. The respiratory chain in masseter muscle mitochondria functioned actively at 5 and 6 weeks of age in klotho mutant mice, but the enzyme activities and the expression of CAT mRNA were lower in klotho mutant mice than in wild-type mice at 7 weeks of age.

Our results also show that changing morphological features (i.e., decreased numbers of cristae in mitochondria) may be related to the induction of mitochondrial enzyme activity in the inner mitochondrial membrane in the masseter muscles of klotho mutant mice. TEM revealed that there were large mitochondria with loose regions of cristae present in klotho mutant mice at 7 weeks of age. Recently, van der Bliek (2009) reported a relationship between mitochondrial fusion and the release of cytochrome c in response to stress. Our previous studies suggested that number of cristae in mitochondria is related to the activity of respiratory chain enzymes in the rat masseter muscle during aging (Sato et al., 1998). In our results, the rate of mitochondrial cristae number reduced in 7 weeks of age to compare to that of 5 weeks of age. In contrast, the amount of mRNAs for cytochrome c, beta subunits of ATP synthase, catalase and the amount of cytochrome c protein increased in 7-week-old klotho mutant mice. Therefore, the release of cytochrome c can lead to increased mitochondrial fission and to the formation of megamitochondria during aging without changing the expression of other apoptosis markers. There are complex apoptotic pathways with more prominent roles in apoptosis than the cytochrome c-dependent pathway. For instance, cytosolic and nuclear levels of apoptosis-inducing factor (AIF), increased endonuclease G (EndoG), and increased levels of the mitochondrial proteins Bcl-2, Bax, and Bid are associated with caspase-independent apoptosis in the skeletal muscle of older rats (Marzetti et al., 2008). Increased levels of Bax and cytochrome c and increased DNA fragmentation have been observed in aged rat gastrocnemius muscles after 14 days of hindlimb suspension (Siu et al., 2003).
Apoptotic signaling pathways may differ in each type of muscle and these pathways may be accompanied by increased cytochrome $c$ release. In general, mitochondria that fuse to form megamitochondria can become separated by fission and can be divided between daughter cells during cytokinesis. The two newly formed cells contain approximately equal numbers of mitochondria. Increased mitochondrial fission occurs in wild-type mice as in rat masseter muscle (Sato et al., 1998). The research done by Tonderra et al. (2009) indicated that stress-induced mitochondrial hyperfusion might counter stress by optimizing mitochondrial ATP production. The present data demonstrate that the reduced expression of beta subunits of ATP synthase mRNA and of other mRNAs may be regulated by the loss of the klotho protein during aging.

Reference

6) Capel F, Buffiere C, Patureau Mirand P and Mosoni L. Differentiations in the temporalis muscles of rabbits after masseter muscle (Sato et al., 1998). The research done by Tonderra et al. (2009) indicated that stress-induced mitochondrial hyperfusion might counter stress by optimizing mitochondrial ATP production. The present data demonstrate that the reduced expression of beta subunits of ATP synthase mRNA and of other mRNAs may be regulated by the loss of the klotho protein during aging.