Klotho Expression is Induced by Calorie Restriction in Adult Male Rats

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Summary

The elderly population in Japan is increasing, and one consequence of aging is a reduction in serum zinc levels. Zinc deficiency reduces the mRNA expression of Klotho, an anti-aging gene which, when disrupted in mice, accelerates the aging process. Although aging is one of the greatest risk factors for disease development later in life, adhering to a calorie-restricted (CR) diet can slow the aging rate and extend life span in many organisms. This study examined the effect of a CR diet on Klotho expression in the kidney of an animal model. Fourteen mature male SD rats were aged until they weighed ~500 g each and were then housed in individual cages and fed a special diet consisting of 65% carbohydrate, 20% protein, 10% fat, and 0.01% zinc, ad libitum. After 1 week, the animals were separated into a control group (n = 7; mean body weight, 511.4 ± 13.7 g), in which each rat consumed 150 kcal/day, and a CR group (n = 7; mean body weight, 529.3 ± 17.2 g), in which each rat consumed 60% of the control calorie intake. After 4 weeks, mean body weight in the CR group was reduced by 13% compared with that in the control group. Serum zinc levels were significantly lower in the CR group (109.7 ± 11.3 µg/dL) compared with the control (132.7 ± 49 µg/dL). Western blot analysis revealed significantly increased Klotho expression in the CR group (0.57 ± 0.05) compared with the control (0.28 ± 0.05). These results suggest that Klotho expression is induced by calorie restriction and is inversely related to serum zinc levels.

Key words: Klotho, Calorie restriction, Serum zinc, Kidney, rat

Introduction

Worldwide, Japan ranks near the top in terms of longevity. Indeed, the Ministry of Health, Labor, and Welfare of Japan announced in 2009 that the average life expectancy of Japanese men and women was 79.6 and 86.4 years, respectively. More than 23% of Japanese citizens are now more than 65 years old. Modern life is characterized by an increase in human longevity, which is accompanied by an increase in aged-related diseases1,2. Aging is one of the greatest risk factors for many diseases associated with aged populations, including cancer, cardiovascular disease, stroke, chronic kidney disease, and neurodegenerative disorders3, which are leading causes of death and disability in aging societies. Suppression of the aging process, if possible, could reduce mortality and improve quality of life. In aging populations, one of the main epidemics of the twenty-first century is metabolic syndrome4. This syndrome includes a cluster of symptoms such as abdominal obesity, dyslipidemia, glucose intolerance, and insulin resistance, as well as premature development of age-related diseases such as type II diabetes, hypertension, and generalized inflammation, and a propensity for developing neurodegenerative diseases.

For more than 70 years, it has been known that dietary calorie restriction slows the rate of aging and extends the life span of many organisms, including yeast and rodents5. Moreover, rodents fed a calorie-restricted (CR) diet display a spectrum of phenotypes that are the direct opposite of those seen in metabolic syndrome, including decreased total body fat, LDL-cholesterol, free fatty acids, and triglycerides; improved glucose tolerance;
and increased HDL-cholesterol\textsuperscript{6}). Additionally, a CR diet delays the development of many age-related disorders such as cancer, diabetes, and neurodegenerative disease\textsuperscript{7,8}). It is therefore possible that the consequences of metabolic syndrome versus calorie restriction lie at diametrically opposing ends of the same spectrum and involve an overlapping set of regulators.

Obesity is a major health problem among the middle aged. Health professionals have made efforts to inform the public about the adverse health consequences of obesity and have emphasized the importance of being “slim.” However, such health education programs can be dangerous to some populations such as young women, who may strive to be thin to such an extent that they develop nutritional disorders during pregnancy, irregular menstruation, and eating disorders\textsuperscript{9}). This degree of caloric restriction may affect various biological parameters, including serum levels of zinc, an important trace element. Zinc deficiency can result in alopecia and compromised immune function.

One gene that has been implicated in aging and age-related metabolic diseases is Klotho, an autosomal recessive gene first identified in a mouse strain that exhibited a syndrome resembling human aging. This strain was generated during an attempt to make transgenic mice and was named after the Greek goddess Klotho, who spins the thread of life. Mice with defective Klotho expression show no visible phenotype in the first 3–4 weeks of life, but subsequently exhibit multiple aging-related phenotypes\textsuperscript{10}, including growth retardation, hypogonadism, thymic involution, skin atrophy, sarcopenia, vascular calcification, osteopenia\textsuperscript{11}, pulmonary emphysema\textsuperscript{12}, cognition impairment, and motor neuron degeneration\textsuperscript{13}). These mice tend to die at around 2 months of age. In comparison, transgenic mice overexpressing Klotho live longer than wild-type mice. Therefore, Klotho may function as an anti-aging gene that extends life span when overexpressed and accelerates aging when disrupted.

Previously, it was reported that zinc deficiency in a rat model decreased Klotho mRNA expression\textsuperscript{14}). Therefore, the present study examined the impact of altered zinc levels on the expression of Klotho in a CR rat model.

### Materials and Methods

1. **An animal model of calorie restriction**

Fourteen male Sprague-Dawley rats (100 g each, five week-old) obtained from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) were provided a standard rat diet (MF of 12-mm pellet type diet, Oriental Yeast Co., Ltd., Tokyo, Japan) \textit{ad libitum} for 2 months, after which the animals’ weights had increased to 500–550 g each. The rats were then divided into two groups and kept in individual cages. Each rat in the control group (n = 7) was fed 25 g of rat chow per day, and each rat in the CR group (n = 7) was provided 15 g of rat chow per day, or 60% of the calorie intake in the control group. After 4 weeks, the animals were put under deep ether anesthesia, and their kidneys were removed for protein and mRNA extraction. The animals were then sacrificed, and their blood was collected for serum mineral analysis.

2. **Measurement of the serum zinc level**

The serum zinc level was measured in the blood samples collected from the rats 4 weeks after the consumption of the zinc-deficient or control diet and was determined by atomic absorption spectrophotometry (Z-6100; Hitachi Co., Tokyo, Japan), as described previously\textsuperscript{15}.

3. **Analysis of Klotho mRNA expression**

Total RNA was isolated according to the protocol recommended by the manufacturer of the real-time PCR machine (ISOGEN, Molecular Research Center Inc., Cincinnati, OH, USA) and was used for cDNA synthesis. Real-time PCR was performed as previously reported\textsuperscript{16}.

4. **Detection of Klotho protein**

Klotho protein and β-actin (internal control) were detected by Western blot analysis. For the detection of Klotho, a piece of kidney cortex (50–70 mg) was homogenized in extraction buffer [HEPES, pH 7.5, 2% CHAPS, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Cat# p8340, Wako Pure Chemical Industries Ltd., Osaka, Japan)] at 4 °C, and the lysate was clarified by centrifugation. The proteins in samples of the supernatant were separated by 7.5% SDS-PAGE and transferred to PVDF membranes (Millipore Co., Bedford, MA, USA). The membranes were blocked with 5% BSA and then incubated with anti-Klotho antibodies (1 μg/mL IgG) in TBS buffer (50 mM Tris-HCl buffer, pH 7.9, 0.1% Tween 20, and 150 mM NaCl) containing 5% BSA overnight at 4 °C. After three washes with TBS buffer, the membranes were incubated with peroxidase-conjugated goat anti-IgG antibodies (1: 2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h with shaking. Bands were visualized by incubation with enhanced chemiluminescence detection reagent (Amer sham), followed by exposure to radiographic film.

For the detection of β-actin as a internal control, the Klotho-probed membranes were stripped of antibody by
incubation in stripping buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) for 30 min at 50°C, washed three times with TBS, blocked with 5% BSA in TBS for 3 h at room temperature, incubated with rabbit anti-actin antibodies (1: 2,000; Sigma Chemicals), and then incubated with peroxidase-conjugated anti-rabbit secondary antibodies (1: 2,000) for 1 h at room temperature. Bands were visualized as described above. Band intensities were analyzed using a CCD image sensor (AE-6920-MF densitograph; ATTO, Tokyo, Japan). Klotho expression is given as the intensity ratio of Klotho to β-actin.

5. Statistical analysis

All results are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using analysis of variance (ANOVA) or Student’s t-test. Differences with p values <0.05 were considered significant.

Results

Over the course of the experiment, the mean body weight of the rats in the CR group gradually decreased, from 493.3 ± 11.1 g at 2 weeks, to 478.7 ± 10.3 g at 3 weeks, and then to 479.3 ± 9.6 g at 4 weeks (Fig. 1). In contrast, the mean weight in the control group gradually increased, from 514.4 ± 13.4 g at 2 weeks, to 523.2 ± 13.5 g at 3 weeks, and then to 548.8 ± 12.1 g at 4 weeks. The difference in weight between the two groups was significant at 2 and 4 weeks (p <0.001).

![Fig. 1](image1)

Fig. 1 Changes in body weight during calorie restriction. Closed circles indicate the calorie-reduced (CR) group; open circles indicate the control group. Values are expressed as means ± SEM. Significant differences are indicated by *p <0.05.

It was demonstrated previously that a decrease in the serum zinc level could reduce Klotho mRNA expression; thus, serum zinc levels were measured (Fig. 2). The mean serum zinc concentration was lower in the CR rats than in the control rats (109 ± 11 vs. 132 ± 5 µg/dL, respectively).

Real-time PCR analysis revealed significantly more Klotho mRNA expression in the CR rats, with an expression level 68% greater than that in the control rats (Fig. 3).

Western blots of Klotho and β-actin protein expression
dominal obesity, improve hypertension, and reduce the risk for mortality from cardiovascular disease. It may also have an anti-aging effect.

The Ministry of Health, Labour, and Welfare of Japan recently announced that approximately 50% of Japanese men and women 40 or more years of age may have lifestyle-related diseases. Given that obesity is a risk factor for lifestyle-related diseases such as type II diabetes mellitus and cardiovascular disease, adoption of a CR diet to reduce obesity or improve weight control may play a significant role in preventing lifestyle-related diseases.

ACR diet can induce the delayed development of autoimmunity and deficiencies in micronutrients such as zinc.\[16, 17]\ It was previously reported that zinc deficiency symptoms such as alopecia appeared when serum zinc decreased to levels of 31 to 38 μg/dL (35.6 ± 1.1 μg/dL) after the intake of a zinc-deficient diet for 4 weeks\[14\]. In the present study, the serum zinc level was significantly lower in the CR group compared with the control group. Although zinc can be both acutely and chronically toxic\[12\], its toxicity is generally disregarded.

Further research is needed to understand the nutritional consequences of a calorie-restricted diet, as well as its risks, especially in young women and fetuses, and its benefits for the prevention of lifestyle-related diseases in middle-aged people.

Fig. 4 Representative Western blot showing Klotho and β-actin immunoreactive bands. Typical bands of Klotho protein and β-actin were shown.

![Western blot image](image)

Fig. 5 Densitometric analyses of Klotho and β-actin protein expression in the control and calorie-reduced (CR) groups. Statistically significant difference is indicated.

![Densitometric analysis](image)

is shown in Fig. 4. As estimated by densitometric analysis, Klotho expression was significantly higher in the CR group (by 103%) than in the control group (Fig. 5).

Discussion

The present study shows that maintaining rats on a CR diet for 4 weeks caused a marked increase in the mRNA and protein expression levels of Klotho in the kidneys of the animals. These results suggest that a CR diet may be effective for the prevention of metabolic syndrome and related diseases such as type II diabetes and hypertension. A CR diet has the potential to reduce ab-

References