CMLS, Cell. Mol. Life Sci. 57 (2000) 738–746
1420-682X/00/010738-9 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Endothelial dysfunction in the *klotho* mouse and downregulation of *klotho* gene expression in various animal models of vascular and metabolic diseases

R. Nagai[a,b,*], Y. Saito[a], Y. Ohyama[a], H. Aizawa[a], T. Suga[a], T. Nakamura[a], M. Kurabayashi[a] and M. Kuro-o[c]

[a]Second Department of Internal Medicine, Gunma University School of Medicine, 3-39-22 Showa, Maebashi, Gunma 371-8511 (Japan), Fax + 81 27 220 8150, e-mail: nagai@news.sb.gunma-u.ac.jp
[b]Department of Cardiovascular Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo, 113-8865 (Japan)
[c]University of Texas Southwestern Medical Center, Dallas (Texas, USA)

Abstract. The human aging process is associated with vascular endothelial dysfunction. However, humoral factors which might protect against endothelial dysfucntion during aging have not yet been identified. We recently identified the *klotho* gene as a possible regulator of human aging. In the present study using the *klotho*-deficient heterozygous mouse, we examined whether the Klotho protein is a humoral factor protecting against endothelial dysfunction. We further cloned rat *klotho* cDNA and investigated whether *klotho* mRNA expression in rat kidney is altered under pathological conditions such as hypertension, hyperlipidemia, renal failure, and inflammatory stress. The Klotho protein itself, or its metabolites, promotes endothelial NO production in aorta as well as arterioles, and *klotho* mRNA in kidney is downregulated under sustained circulatory stress.

Key words. *klotho*; endothelial function; nitric oxide production; hypertension; renal failure; cytokine.

Introduction

Risk factors for atherosclerosis, such as hyperlipidemia, hypertension, diabetes mellitus, and cigarette smoking, induce endothelial dysfunction as revealed by reduced endothelial production of nitric oxide (NO) or endothelial expression of cell adhesion molecules. Patients with the four major risk factors develop atherosclerosis at a higher frequency, but there are many atherosclerotic patients without these factors, suggesting the presence of other unknown risk factors regulating the development of atherosclerosis, of which the most important 'aging'. We recently identified the *klotho* gene as a possible regulator of the human aging process [1]. Because endothelial function is impaired in aged subjects, the Klotho protein may plausibly be hypothesized as protecting against endothelial dysfunction. In the present study, we examined whether *klotho*-deficient mice have endothelial dysfunction and *klotho* mRNA expression in kidney is downregulated under circulatory stress.

* Corresponding author.

We first found that the *klotho* gene product itself, or its metabolites, protects against endothelial dysfunction as a humoral factor because endothelium-dependent arterial dilatation was impaired in the *klotho*-deficient heterozygous mouse, and was improved by establishing parabiosis with a wild-type mouse but not with a heterozygous mouse [2]. NO production was also reduced in heterozygous *klotho* mice compared to wild-type mice. We have also shown that in animal models of hypertension, renal disease, hyperlipidemia, and diabetes, *klotho* mRNA levels were reduced in the kidney, suggesting that *klotho* gene expression is regulated by circulatory or metabolic stress against the kidney [3, 4].

Materials and methods

Animals

All procedures were in accordance with institutional guidelines for animal research. Mice and rats were housed two or three per cage and maintained under controlled conditions of light, temperature, and humidity.

***klotho* mouse.** Heterozygous *klotho* mice were maintained in a specific-pathogen-free animal facility at Gunma University School of Medicine. DNA was extracted from the tail, and screened for deletion of the *klotho* gene by the polymerase chain reaction (PCR). Homozygous or heterozygous *klotho* mice 6–8 weeks old were used for experiments.

Parabiosis. Surgery was carried out on *klotho* mice (6–8 weeks old) under pentobarbital anesthesia (30 mg/kg). A lateral incision was made from the foreleg to the gluteal muscle along the adjacent sides of each mouse. The adjacent muscles on the lateral side of the peritoneum of two mice were divided and the abdominal muscles on the right side of one animal were sutured to the abdominal muscles on the left side of the other animal so that peritoneal surfaces were connected. The two mice were sutured skin to skin with ventral and dorsal skin opposed [5]. Two or 4 weeks after surgery, the vasodilator responses of aorta and arterioles to acetylcholine were investigated.

SHR. Male SHR and Wistar-Kyoto (WKY) rats were purchased at 6 weeks, and fed standard laboratory chow and given tap water ad libitum. Rats were sacrificed at 6, 18, and 60 weeks (n = 3–6 in each group).

Deoxycorticosterone acetate salt hypertensive rats. A total of 16 male Wistar rats weighing approximately 200 g were used. Deoxycorticosterone acetate (DOCA) salt hypertensive rats were prepared by a previously described method [6]. Briefly, all rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg), and were unilaterally nephrectomized. At 7 days, eight rats were subcutaneously implanted with a pellet of DOCA (25 mg) near the dorsal rib cage, and received a second implant of DOCA 14 days later, and a third implant, another 14 days later. The remaining eight rats were sham-operated three times without receiving DOCA implants. DOCA-implanted rats were fed standard laboratory chow and given salt solution (17.1 mEq NaCl + 2.7 mEq KCl/100 ml water). Control rats were fed standard laboratory chow and given water not containing NaCl or KCl. All rats were sacrificed 14 days after the third operation.

Five-sixths-nephrectomized rats. A total of 20 male Wistar rats weighing approximately 150 g were used for the present study. All rats were anesthetized with sodium pentobarbital, and 10 underwent 2/3 nephrectomy of the left kidney, and 7 days later, total nephrectomy of the right kidney. The remaining 10 rats were sham-operated without nephrectomy (controls). Rats were sacrificed at 4 and 8 weeks after total nephrectomy or sham operation (n = 5 in each group).

OLETF rats. Male OLETF and Long-Evans Tokushima Otsuka (LETO) rats (control strain) at 6 weeks were a kind gift from the animal resources of Otsuka Pharmaceuticals, Tokushima, Japan, and were bred in our animal laboratory facilities. They were fed standard laboratory chow and given tap water ad libitum. These rats were sacrificed at 40 weeks (n = 9 in each group).

Rats with myocardial infarction. Male Wistar rats weighing 250–350 g were used for the present study. Myocardial infarction (MI) was produced by a previously described method [7]. Rats were fed standard laboratory chow and given tap water ad libitum, and sacrificed at two time points: 4 and 7 days after coronary ligation or sham operation (n = 5 in each group).

Endothelial function in the *klotho* mouse

Aortic ring experiments. *klotho* mice (6–8 weeks old) were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg). The chest wall was opened, and the descending thoracic aorta was carefully removed to avoid damaging endothelium. After blood and connective tissues had been removed, a cylindrical segment (3 mm long) was excised from the aorta. The aortic ring segment was mounted between two stainless steel wires in 10 ml of organ bath containing Krebs' bicarbonate solution bubbled with a mixture of 95% O_2 and 5% CO_2 to obtain rapid mixing of drugs. The composition of the Krebs' bicarbonate solution was 120 mM NaCl, 5.2 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 0.03 mM Na_2-EDTA, and 11 mM dextrose (pH 7.4). One wire was attached to a fixed support, and the other was connected to a force-displacement transducer (model UR-50G; Minebea, Nagano, Japan) [8]. To test whether endothelial function is impaired in apparently normal arteries in heterozygous *klotho* mice, we examined acetylcholine-induced relaxation of aortic rings isolated from wild-type (n = 7; male 3, female 4) and heterozygous *klotho* mice (n = 7; male 4, female 3). The rings were suspended under 0.93 g of tension. The preparation was allowed to equilibrate for 90 min and preconstricted by norepinephrine (10^{-7} M). To obtain a dose-response curve for acetylcholine (10-8 to10-5 M) and sodium nitroprusside (10^{-10}–10^{-7} M) stock solution of drugs was added cumulatively in an organ bath. Data were expressed as percentage relaxation of norepinephrine-induced preconstriction. Aortic rings obtained from homozygous *klotho* mice showed a complete absence of constriction or dilatation in response to norepinephrine or acetylcholine due to severe calcification.

Microcirculation study. In addition to studies using aorta, we analyzed endothelial function of the microcirculation in homozygous *klotho* mice (6–8 weeks old), as calcification was not evident in arterioles of skeletal muscle. Male wild-type mice (n = 7), heterozygous mice

(n = 7), and homozygous mice (n = 7) were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg). Esophageal temperature was maintained at 37–38 °C by a servo-controlled heating pad. The trachea was intubated. Under stereomicroscopy, the scrotum was separated from the muscle. The edge of the cremaster muscle was secured with insect pins. During the dissection and experimental period, the cremaster muscle was superfused continuously with warm physiological solution (34 °C, pH 7.35) of the following composition: 132 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 20 mM $NaHCO_3$. The superfusion solution was equilibrated with gas containing 5% CO_2 and 95% N_2 [5].

The cremaster muscle preparation was transferred to an intravital microscope (Optiphoto; Nikon, Tokyo, Japan) and equilibrated for 30 min. Microvessels were observed using an illuminated microscope (N.A. = 0.40). A CCD camera (model C-2400-77; Hamamatsu, Shizuoka, Japan) positioned on an imaging tube was coupled to a video monitor (model PVM 1343; Sony, Tokyo, Japan). The final magnification of the monitor was × 800. The vessel diameter was measured with a video analyzer (Argus-10; Hamamatsu, Shizuoka, Japan). The resolution of this system was ± 1 mm. We examined whether the vasodilator response of arterioles to topical application of acetylcholine is attenuated in the cremaster muscle of *klotho* mice. To obtain a concentration-response curve for acetylcholine (10^{-8}–10^{-5} M) and sodium nitroprusside (10^{-9}–10^{-6} M), the stock solution of drugs was added cumulatively to superfusion solution. Data were expressed as percentage dilatation of the resting diameter.

Measurement of NO_2^- and NO_3^- in urine. We measured urinary excretion of NO_2^- and NO_3^- in heterozygous *klotho* mice (6–8 weeks old) to examine whether systemic NO production is decreased. Wild-type and heterozygous *klotho* mice (n = 13) were housed individually in metabolic cages. To evaluate endogenous NO production, mice were administered the NO synthesis inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME; 500 mg/l in drinking water) for 2 weeks. Oral treatment with L-NAME for 2 weeks has been demonstrated to completely inhibit endogenous NO production [9]. The concentration of NO_2^- and NO_3^- in urine was measured by an autoanalyzer (TCI-NOX 1000; Tokyo Kasei Kogyo, Tokyo, Japan). Deproteinized urine samples premixed with the carrier solution (0.007% EDTA and 0.03% NH4Cl) were passed through a cadmium reduction column covered with copper to reduce NO_2^- and NO_3^- which react with Griess reagent (1% sulfonamide and 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% HCl) to form a purple azo dye. Absorbance was detected at 540 nm using a flow-through visible spectrophotometer (model S/3250; Soma-Kogaku, Tokyo, Japan) connected to a strip chart recorder. The limit of detection of NO_2^- was 0.2 mM (with 99% confidence) and the intra- and inter-assay coefficients of variation were 1.6 and 1.7%, respectively [9, 10].

Blood pressure measurement

In SHR, DOCA-salt hypertensive rats, MI rats, and control rats for each group, systolic blood pressure (SBP) was measured by the photoelectric volume oscillometric method with a UR-5000 automated tail cuff sphygmomanometer (Ueda) without anesthesia. In OLETF and LETO rats, a polyethylene catheter (PE-50) was placed in the right femoral artery under sodium pentobarbital anesthesia (50 mg/kg), and then mean arterial blood pressure was measured through the right femoral catheter by a pressure transducer (model 1829; NEC) and recorded on a physiograph (OMNILIGHT 8M36; NEC). Values are shown as averages of five to ten consecutive measurements.

Blood analysis

Blood samples were withdrawn from the abdominal aorta (SHR, DOCA-salt hypertensive rats, and control rats for each group) or the right femoral artery (OLTEF and LETO rats) under sodium pentobarbital anesthesia. The samples were centrifuged at 3000 rpm for 15 min at 4 °C. After centrifugation, the plasma samples were stored immediately at − 30 °C, and biochemical analysis was done by an automated analyzer (MBC, Gunma, Japan).

Molecular cloning of rat *klotho* cDNA

We isolated rat *klotho* cDNA from a rat lung cDNA library [4]. The rat *klotho* cDNA coding region was 93% and 83% identical to the mouse and human *klotho* cDNA coding regions, respectively [1]. The deduced amino acid sequence of rat Klotho protein was 1014 in length and contained a putative signal sequence at the amino terminus and a single transmembrane domain near the carboxyl terminus, indicative of a type I membrane protein. The deduced amino acid sequence of rat Klotho protein showed 94 and 85% identity to those of mouse and human Klotho proteins, respectively. Rat Klotho protein contained a short stretch of basic amino acids (Lys-Lys-Arg-Lys) between the two internal repeats of the extracellular domain (rKL1 and rKL2), a possible site for proteolytic cleavage similar to the polybasic proteolytic processing site reported in the transforming growth factor-β superfamily [11, 12].

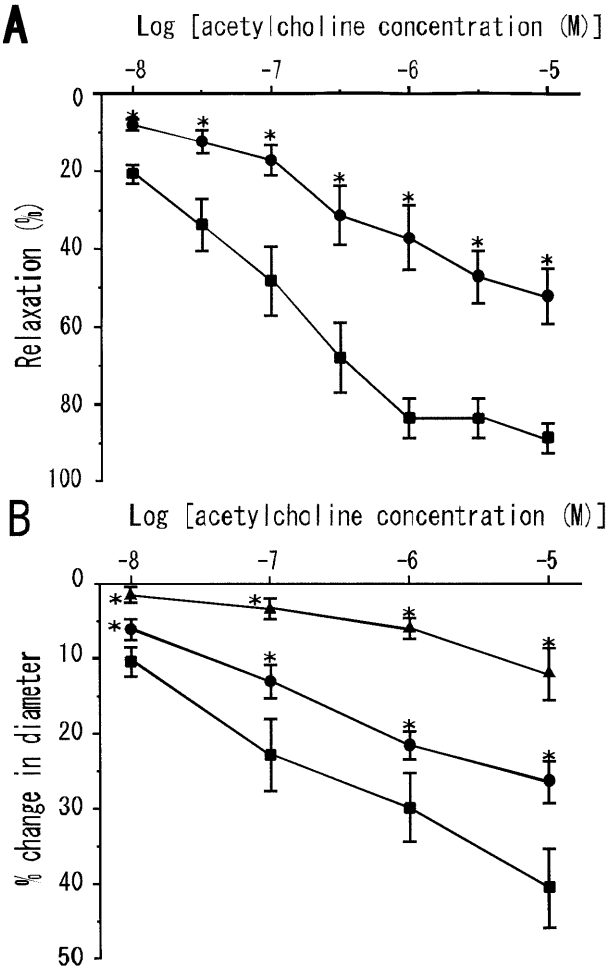

Figure 1A, B. Endothelium-dependent vasodilatation in response to acetylcholine in *klotho* mice. (*A*) Endothelium-dependent relaxation of aorta. The putative maximal relaxation was considered to be the level before preconstriction by norepinephrine. The response to each concentration was expressed as the percentage of the putative maximal relaxation. Squares, wild-type mice (n = 7, male 3, female 4); circles, heterozygous *klotho* mice (n = 7, male 4, female 3). *P < 0.05 compared with wild-type mice by unpaired t-test. (*B*) Endothelium-dependent dilation of arterioles. The change in diameter (%) of arterioles in response to acetylcholine was demonstrated. Squares, wild-type mice (n = 7); circles, heterozygous *klotho* mice (n = 7); triangles, homozygous *klotho* mice (n = 7). *P < 0.05 compared with wild-type mice by ANOVA.

Northern blot analysis

Tissues collected from rats were immediately frozen in liquid nitrogen. Total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method. Poly(A)$^+$ RNA was prepared using Oligotex dT-30 Super (Daiichi Pure Chemicals, Tokyo, Japan). Poly(A)$^+$ RNA (2 μg) was electrophoresed on a 1% agarose gel containing formaldehyde, and transferred to a nylon membrane (Hybond N+, Amersham). The 830-bp fragment of rat *klotho* cDNA (nucleotides 1656–2485) and the 500-bp fragment of rat GAPDH cDNA (nucleotides 1656–2485) were used as probes. Probes were labeled with 32P by random priming, using a commercially available kit (Boehringer Mannheim). Prehybridization and hybridization were carried out according to standard methods at 42 °C in 40% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml of sonicated denatured salmon DNA.

Statistical analysis

Results are presented as the mean ± SE. Data were evaluated by unpaired t-test or analysis of variance (ANOVA) for repeated measures. Statistical significance was considered to be $P < 0.05$.

Results

Endothelial dysfunction in the *klotho*-deficient heterozygous mouse

In wild-type and heterozygous *klotho* mice, the cumulative application of acetylcholine produced endothelium-dependent relaxation of aortic rings preconstricted with norepinephrine. However, the maximum relaxation induced by acetylcholine was significantly attenuated in heterozygous *klotho* mice (53 ± 7%) compared with wild-type mice (88 ± 4%, fig. 1A; $P < 0.05$). The sensitivity (ED_{50}) of acetylcholine-induced relaxation was significantly greater in heterozygous *klotho* mice (4×10^{-5} M) than in wild-type mice (8×10^{-6} M; table 1; $P < 0.05$). There was no significant difference in aortic relaxation in response to sodium nitroprusside between wild-type and heterozygous *klotho* mice (table 1).

Table 1. Sensitivity (ED_{50}) of aortic ring segments to acetylcholine or sodium nitroprusside.

	Wild type	Heterozygote	Parabiosis 2w H+W	Parabiosis 4w H+W	Parabiosis 4w H+H
Acetylcholine	6.9 ± 0.2	5.6 ± 0.3*	6.2 ± 0.4	7.0 ± 0.5	5.3 ± 0.2*
Sodium nitroprusside	8.7 ± 0.2	8.7 ± 0.2	8.8 ± 0.1	9.1 ± 0.5	8.9 ± 0.2

Aortic rings were isolated from heterozygous mice in parabiosis to wild-type mice (H+W) or heterozygous mice (H+H) after 2 weeks (2w) and 4 weeks (4w) of parabiotic surgery. The ED_{50} indicates the negative logarithm of 50% relaxation. Values are the mean ± SE. *P < 0.05 compared with wild type.

Table 2. Sensitivity (ED_{20}) of arterioles to acetylcholine or sodium nitroprusside.

	Wild type	Heterozygote	Homozygote	Parabiosis H+W	Parabiosis H+H
Acetylcholine	7.1 ± 0.3	6.3 ± 0.3*	<5*	7.1 ± 0.3	5.3 ± 0.1*
Sodium nitroprusside	7.7 ± 0.3	7.9 ± 0.4	7.8 ± 0.4	N/A	N/A

Arteriolar vasodilatation in response to acetylcholine was observed in heterozygous mice in parabiosis to wild-type mice (H+W) or heterozygous mice (H+H) after 4 weeks of parabiotic surgery. The ED_{20} indicates the negative logarithm of 20% dilation from resting diameter. N/A, not available. Values are the mean ± SE. *$P < 0.05$ compared with wild type.

Arteriolar vasodilatation in response to acetylcholine and sodium nitroprusside

The diameters of the arterioles before application of acetylcholine were 49 ± 5, 43 ± 4, and 33 ± 3 μm in wild-type, heterozygous *klotho* mice, and homozygous *klotho* mice, respectively. Arterioles dilated in response to acetylcholine in a concentration-dependent manner in all mice. However, the maximum response to acetylcholine was significantly attenuated in homozygous ($12 \pm 3\%$) and heterozygous *klotho* mice ($27 \pm 3\%$) compared with wild-type mice ($41 \pm 5\%$) (fig. 1B; $P < 0.05$). The sensitivity (ED_{20}) to acetylcholine in homozygous ($> 1 \times 10^{-5}$ M) and heterozygous *klotho* mice (2×10^{-6} M) was also significantly greater than in wild-type mice (1×10^{-7} M) (table 2; $P < 0.05$). There was no significant difference in vasodilatation induced by sodium nitroprusside among these groups (table 2).

Excretion of NO_2^- and NO_3^- in urine is reduced in the heterozygous *klotho* mouse

Urinary excretion of NO_2^- and NO_3^- was significantly lower in heterozygous *klotho* mice (142 ± 16 nmol/day) than in wild-type mice (241 ± 28 nmol/day; fig. 2; $P < 0.05$). After treatment with L-NAME, by administration in drinking water for 2 weeks, urinary excretion of NO_2^- and NO_3^- was significantly decreased in both wild-type and heterozygous *klotho* mice, and no longer differed between the two mice genotypes.

Parabiosis with wild-type mouse improves reduced endothelial NO production in the heterozygous *klotho* mouse

Sixty-seven out of 88 pairs developed parabiotic disharmony within 2 weeks. After 2 weeks of parabiosis, relaxation of aortic rings in response to 10^{-5} M acetylcholine was improved in parabiotic heterozygous *klotho* mice. Surprisingly, no significant difference was observed in aortic relaxation in response to acetylcholine between parabiosed heterozygous *klotho* mice and wild-type mice after 4 weeks of parabiosis (fig. 3A; $P < 0.05$). Furthermore, endothelial function of arterioles in heterozygous *klotho* mice was also restored after 4 weeks of parabiosis to wild-type mice (fig. 3B). On the other hand, parabiosis of heterozygous pairs failed to normalize acetylcholine-induced vasodilatation of aorta and arterioles (tables 1, 2).

klotho mRNA expression in kidney is reduced in rat models of hypertension, renal failure, and hyperlipidemia

SHR. Body weight, SBP and wet tissue weight of the kidney to body weight in 6-, 18- and 60-week-old SHR and WKY rats are summarized in table 3. The SBP of SHR was statistically higher than age-matched WKY rats ($P < 0.01$). No difference was observed between SHR and WKY rats with wet tissue weight of the kidney to body weight in 18- and 60-week-olds, although this ratio in SHR was greater in 6-week-olds. *klotho* mRNA levels in the kidney of WKY rats were

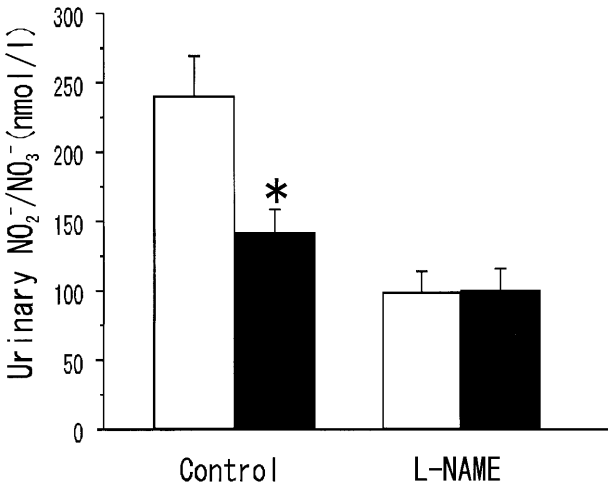

Figure 2. Urinary excretion of NO_2^- plus NO_3^- before and after N^{ω}-nitro-L-arginine methyl ester (L-NAME) treatment orally for 2 weeks in wild-type mice (open bars) and heterozygous mice (closed bars). After L-NAME treatment, wild-type and heterozygous mice did not differ ($n = 13$). *$P < 0.05$ compared with wild-type mice by unpaired t-test.

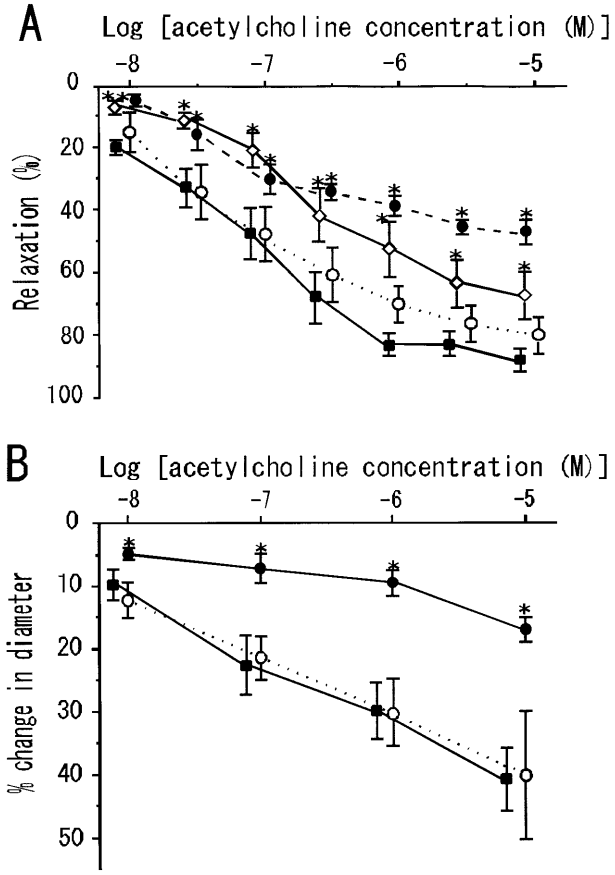

Figure 3A, B. Endothelium-dependent vasodilatation in response to acetylcholine after parabiosis. (*A*) Acetylcholine-induced relaxation of aortic rings obtained from heterozygous mice in parabiosis to wild-type or heterozygous mice. Open circles, heterozygous *klotho* mice after 4 weeks of parabiosis to wild-type mice (n = 5, male 2, female 3); open diamonds, heterozygous *klotho* mice after 2 weeks of parabiosis to wild-type mice (n = 6, male 2, female 4); closed circles, heterozygous *klotho* mice after 4 weeks parabiosis to heterozygous *klotho* mice (n = 6, male 4, female 2), squares, wild-type mice (n = 7). *$P < 0.05$ compared with wild-type mice by ANOVA. (*B*) Endothelium-dependent dilation of arterioles after parabiosis. The change in diameter (%) of arterioles in response to acetylcholine is shown. Open circles, heterozygous *klotho* mice after 4 weeks of parabiosis to wild-type mice (n = 4); closed circles, heterozygous *klotho* mice after 4 weeks of parabiosis to heterozygous *klotho* mice (n = 4), squares, wild-type mice (n = 7). *$P < 0.05$ compared with wild-type mice by ANOVA.

unchanged in 6-, 18- and 60-week-olds (data not shown). Quantitatively, *klotho* mRNA levels in the kidney of SHR in 6- and 18-week-olds were not significantly lower than in age-matched WKY rats, but were significantly lower in 60-week-olds than WKY rats (fig. 4).

DOCA salt hypertensive rats. Body weight, SBP, and wet tissue weight of the kidney to body weight of DOCA salt hypertensive rats and sham-operated rats are summarized in table 3. Body weight of DOCA salt hypertensive rats were significantly lower than sham-operated rats ($P < 0.01$). The SBP of DOCA salt hypertensive rats was statistically higher than in sham-operated rats ($P < 0.01$). Wet tissue weight of the kidney to body weight of DOCA salt hypertensive rats was significantly greater than in sham-operated rats ($P < 0.01$). Serum cholesterol (T-chol) of DOCA salt hypertensive rats was significantly lower than in sham-operated rats (79 ± 7 vs 154 ± 23 mg/dl, $n = 8$, $P < 0.05$). Blood glucose (BG) of DOCA salt hypertensive rats was significantly higher than in sham-operated rats (199 ± 10 vs 150 ± 10 mg/dl, $n = 8$, $P < 0.01$). DOCA salt hypertensive rats of this stage showed lower expression levels of *klotho* mRNA in the kidney than did sham-operated rats (fig. 5).

Five-sixths-nephrectomized rats. *klotho* mRNA levels in the kidney of 5/6-nephrectomized rats at 4 weeks post operation were lower than in sham-operated rats, although the difference did not reach statistical significance. At 8 weeks post-operation, *klotho* mRNA levels in the kidney of 5/6-nephrectomized rats were significantly lower than in sham-operated rats (fig. 6).

OLETF rats. The body weight of OLETF rats was significantly higher than that of LETO rats (628 ± 23 vs 465 ± 17 g, $n = 8$, $P < 0.01$). The mean blood pressure of OLETF rats was statistically higher than that of LETO rats (115 ± 3.7 vs 93 ± 2.4 mmHg, $n = 8$, $P < 0.01$). No difference was observed between OLETF and LETO rats with respect to wet tissue weight of the kidney to body weight (0.31 ± 0.01 vs $0.28 \pm 0.01\%$, $n = 8$). BG, T-chol and triglyceride (TG) of OLETF rats were significantly higher than in LETO rats at 40 weeks (BG: 329 ± 36 vs 159 ± 14 mg/dl; T-chol: 177 ± 10 vs 98 ± 2 mg/dl; TG: 353 ± 45 vs 77 ± 11 mg/dl, $n = 9$, $P < 0.05$). OLETF rats at this stage showed lower levels of *klotho* mRNA in the kidney than age-matched LETO rats (fig. 7).

Discussion

In the present study, we demonstrated that relaxation of aortic rings ex vivo as well as that of intramuscular arterioles in vivo is impaired in *klotho* mice in response to acetylcholine. We further demonstrated that parabiosis between heterozygous *klotho* mouse with wild-type mouse results in complete recovery of endothelial dysfunction in *klotho* mice 4 weeks after surgery, suggesting that the Klotho protein itself or its metabolites may function as a humoral factor promoting endothelial NO production.

Human aging is associated with impaired endothelium-dependent dilatation of blood vessels [13]. Vasodilatation in response to acetylcholine in apparently normal coronary arteries is progressively impaired with aging in humans [13]. A similar phenomenon has also been demonstrated in the forearm; that is, an endothelium-

Table 3. Characteristics of SHR, DOCA-salt hypertensive rats and control rats.

		BW (g)	SBP (mmHg)	Kidney/BW (g%)
6 weeks	WKY (n = 6)	129 ± 0.6	126 ± 3.5	0.54 ± 0.01
	SHR (n = 6)	126 ± 1.6	149 ± 3.9*	0.62 ± 0.02†
18 weeks	WKY (n = 6)	339 ± 3.0	158 ± 5.4	0.31 ± 0.005
	SHR (n = 6)	332 ± 3.8	247 ± 9.3*	0.32 ± 0.01
60 weeks	WKY (n = 3)	362 ± 27	132 ± 5.8	0.39 ± 0.02
	SHR (n = 6)	356 ± 13	227 ± 11*	0.40 ± 0.01
Sham (n = 8)		672 ± 28	139 ± 4.3	0.48 ± 0.02
DOCA (n = 8)		389 ± 32‡	223 ± 6.8‡	0.94 ± 0.09‡

BW, body weight; SBP, systolic blood pressure; Kidney/BW, wet tissue weight of the kidney to 100 g body weight. Values are expressed as the mean ± SE.
* $P < 0.01$, SHR vs WKY rats within the same age group.
† $P < 0.05$, SHR vs WKY rats within the same age group.
‡ $P < 0.01$, DOCA-salt hypertensive rats vs sham-operated rats.

dependent response to acetylcholine decreased with advancing age in both normotensive and hypertensive subjects [14]. Egashira et al. [15] have demonstrated that endothelium-dependent vasodilatation of coronary microvasculature in response to acetylcholine was attenuated by aging in humans. Therefore, impaired vasodilatation in response to acetylcholine possibly reflects an abnormality in the endothelial cell, which is considered to be an important factor in the development of arteriosclerosis.

Endothelial dysfunction in *klotho* mice may be due to endogenous endothelial NO production. Urinary excretion of the NO metabolites, NO_2^- and NO_3^-, in heterozygous *klotho* mice is significantly decreased compared with wild-type mice. However, urinary NO_2^- and NO_3^- are derived not only from endogenous NO products but also from exogenous NOx included in drinking water and mouse chow. Nevertheless, when we treated mice with L-NAME for 2 weeks, urinary excretion of NO_2^- and NO_3^- did not differ between heterozygous *klotho* mice and wild-type mice, suggesting endogenous NO production to be significantly decreased in heterozygous *klotho* mice. This indicates that the *klotho* mouse may be a novel animal model useful for investigating mechanisms regulating endothelial NO production and atherogenesis.

The *klotho* gene is mainly expressed in specific cells and tissues such as renal tubules and choroid plexus [1]. RT-PCR detected expression of the *klotho* gene in vascular smooth muscle, but not in vascular endothelium. In situ hybridization failed to detect *klotho* gene expression in organs with severe age-related phenotypes in homozygous *klotho* mice, including bone, lung, and skin. These findings suggest that *klotho* gene products function as a humoral factor. To clarify this, we examined whether endothelial dysfunction in *klotho* mice can be improved by parabiosis with wild-type mouse, which should establish exchange of blood plasma after 2 weeks of surgery [16]. Our parabiosis results clearly indicated that aortic relaxation in response to 10^{-5} M acetylcholine was significantly improved in heterozygous *klotho* mice after 2 weeks of parabiosis to wild-type mice. Furthermore, the vasodilatory response to acetylcholine was normalized after 4 weeks of parabiosis. Parabiosis of a heterozygous *klotho* mice pair did not show a significant effect on vasodilator responses to acetylcholine, demonstrating that an improvement of acetylcholine-induced vasodilatation was not due to nonspecific effects of parabiosis. These results suggest that the Klotho protein itself or related circulating factor(s) in wild-type mice can be transmitted to heterozygous *klotho* mice by successful parabiosis, restor-

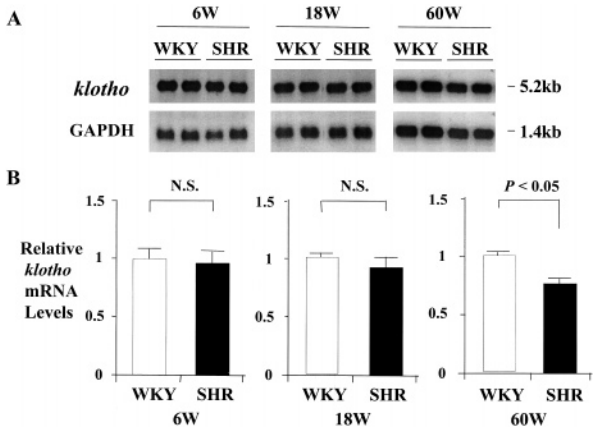

Figure 4A, B. *klotho* mRNA expression levels in kidney isolated from 6-, 18-, and 60-week-old SHR and age-matched WKY rats. (*A*) Autoradiograms of Northern blot analysis of poly(A)$^+$ RNA (2 mg) from the kidney for *klotho* and GAPDH mRNAs. (*B*) Summary of the quantitative changes in *klotho* mRNA expression levels. Values are expressed as the mean ± SE (60-week-old WKY rats, n = 3; others, n = 6).

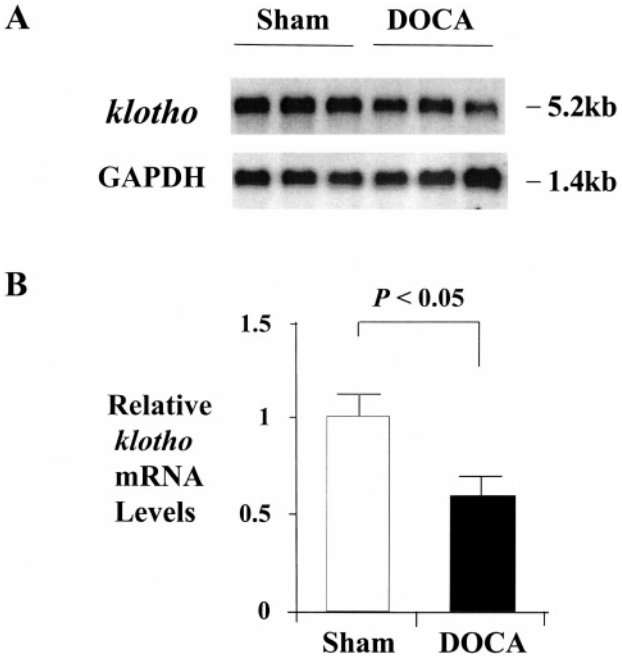

Figure 5A, B. *klotho* mRNA expression levels in kidney isolated from DOCA salt hypertensive rats and sham-operated rats. (*A*) Autoradiograms of Northern blot analysis of poly(A)$^+$ RNA (2 mg) from the kidney for *klotho* and GAPDH mRNAs. (*B*) Summary of the quantitative changes in *klotho* mRNA expression levels. Values are expressed as the mean ± SE (n = 5).

ing endothelial function. We suggest that deficiency of the Klotho protein is either directly or indirectly involved in a decrease in NO production. The mechanism of NO synthesis mediated by the Klotho protein is as yet unknown and is a future research target.

In the second part of this study, we demonstrated that expression of the *klotho* gene in the kidney is downregulated in rats with pathological conditions, such as hypertensive rats, 5/6-nephrectomized rats, and OLETF rats. In SHR, a model of essential hypertension in humans, renal expression of the *klotho* gene was not significantly downregulated in 6- and 18-week-olds, but was significantly downregulated in 60-week-olds. The SBP of SHR reaches hypertensive levels at 6 weeks, continued to increase until 18 weeks, and remained elevated through the life of the rat (16–18 months) [17]. Contrast microscopic study of the kidney of the SHR strain demonstrated no differences from age-mached WKY rats until 20 weeks, but a progressive increase in pathologic lesions was observed from 30 weeks (glomerulosclerosis, protein casts of tubules, intimal proliferation, medial hyperplasia, and fibrinoid degeneration in small arteries and arterioles) [17, 18]. These data suggest that downregulation of *klotho* mRNA is not a causative factor for developing hypertension in SHR, but rather secondary to renal damage from long-term hypertension.

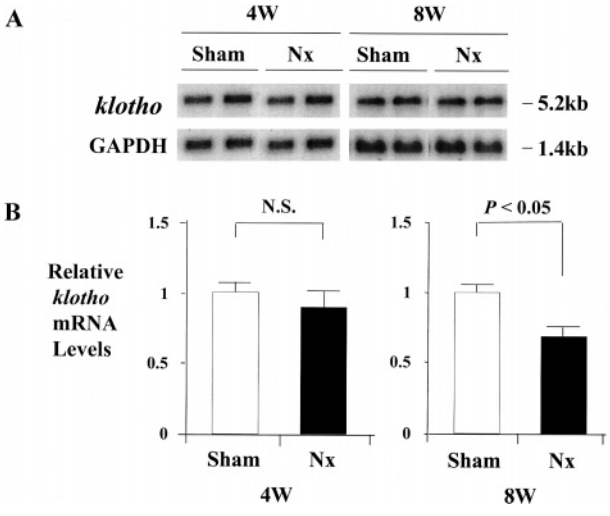

Figure 6A, B. *klotho* mRNA expression levels in kidney isolated 4 and 8 weeks after 5/6 nephrectomy (Nx) and from sham-operated rats. (*A*) Autoradiograms of Northern blot analysis of poly(A)$^+$ RNA (2 mg) from the kidney for *klotho* and GAPDH mRNAs. (*B*) Summary of the quantitative changes in *klotho* mRNA expression levels. Values are expressed as the mean ± SE (n = 5).

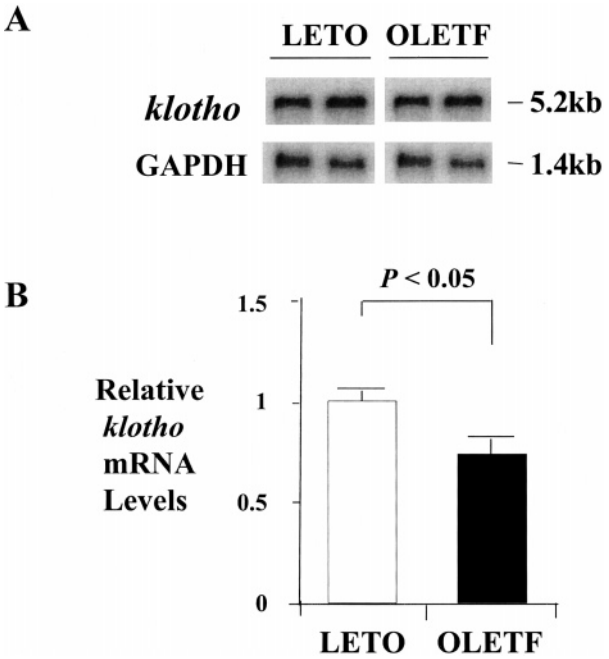

Figure 7A, B. *klotho* mRNA expression levels in the kidney isolated from 40-week-old OLETF rats and age-matched LETO rats. (*A*) Autoradiograms of Northern blot analysis of poly(A)$^+$ RNA (2 mg) from the kidney for *klotho* and GAPDH mRNAs. (*B*) Summary of the quantitative changes in *klotho* mRNA expression levels. Values are expressed as the mean ± SE (n = 6).

Similar changes in *klotho* mRNA levels in the kidney were observed in DOCA salt hypertensive rats, a model of a volume-dependent form of hypertension. Contrast microscopic study of the kidney revealed progressive lesions. Endothelium-dependent relaxation was also attenuated, possibly due to a decrease in NO production from the endothelial cells. These data again suggest that the reduced expression of the *klotho* gene is secondary to renal damage and the Klotho protein is likely involved in the production of NO, either directly or indirectly.

In nephrectomized rats, a model of chronic renal failure in humans, and in OLETF rats, an animal model for human non-insulin-dependent diabetes mellitus, expression of the *klotho* gene in the kidney was also significantly downregulated. Microscopic studies of the kidney of nephrectomized and OLETF rats showed a progressive increase in pathologic lesions [19, 20]. On the other hand, expression of the *klotho* gene in the kidney in rats with myocardial infarction or hypovolemic shock was not different from sham-operated rats, although blood pressure was significantly decreased (data not shown), suggesting that expression of the *klotho* gene in the kidney is not necessarily regulated by transient changes in renal perfusion pressure. Nephrectomized and OLETF rats have been reported to show impaired endothelium-dependent relaxation possibly due to a decrease in NO production from endothelial cells [21, 22]. These data would also indicate that downregulation of the *klotho* gene may be involved in endothelial dysfunction.

Whether the *klotho* gene product might be pharmaceutically useful in preventing cardiovascular disease remains an open question. To answer this, the physiological role of the Klotho protein in the regulation of NO synthesis in normal and pathological animal models must be clarified. Supplementation of the Klotho protein to these animal models should provide answers to these questions.

Acknowledgments. We thank Yoshiko Nonaka, Mayumi Yoshii, Akemi Yoguchi, Shizuko Saiki, Miki Yamazaki, and Kaori Ishihara for their technical assistance. This study was supported by the Japanese Ministry of Education, Science and Culture, and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR).

1 Kuro-o M., Matsumura Y., Aizawa H., Kawaguchi H., Suga T., Utsugi T. et al. (1997) Mutation of the mouse *klotho* gene leads to a syndrome resembling ageing. Nature **390:** 45–51
2 Saito Y., Yamagishi T., Nakamura T., Ohyama Y., Aizawa H., Suga T. et al. (1998) Klotho protein protects against endothelial dysfunction. Biochem. Biophys. Res. Commun. **248:** 324–329
3 Aizawa H., Saito Y., Nakamura T., Inoue M., Imanari T., Ohyama Y. et al. (1998) Downregulation of the *klotho* gene in the kidney under sustained circulatory stress in rats. Biochem. Biophys. Res. Commun. **249:** 865–871
4 Ohyama Y., Kurabayashi M., Masuda H., Nakamura T., Aihara Y., Kaname T. et al. (1998) Molecular cloning of rat *klotho* cDNA: markedly decreased expression of *klotho* by acute inflammatory stress. Biochem. Biophys. Res. Commun. **251:** 920–925
5 Nishizawa Y. and Bray G. A. (1980) Evidence for a circulating ergostatic factor: studies on parabiotic rats. Am. J. Physiol. **239:** R344–R351
6 Tajima Y., Ichikawa S., Sakamaki T., Matsuo H., Aizawa F., Kogure M. et al. (1983) Body fluid distribution in the maintenance of DOCA-salt hypertension in rats. Am. J. Physiol. **244:** H695–H700
7 Kanda T., Nakano M., Arai M., Yamamoto A., Suzuki T. and Murata K. (1993) Myocardial atrial natriuretic factor gene expression after coronary ligation in rats. Exp. Clin. Endocrinol. **101:** 173–177
8 Hoshino J., Sakamaki T., Nakamura T., Kobayashi M., Kato M., Sakamoto H. et al. (1994) Exaggerated vascular response due to endothelial dysfunction and role of the renin-angiotensin system at early stage of renal hypertension in rats. Circ. Res. **74:** 130–138
9 Nakamura T., Ohyama Y., Masuda H., Kurashina T., Saito Y., Kato T. et al. (1997) Antihypertensive agents prevent nephrosclerosis and left ventricular hypertrophy induced in rats by prolonged inhibition of nitric oxide synthesis. J. Hypertens. **15:** 373–381
10 Boger R.H., Bode-Boger S. M., Thiele W., Junker W., Alexander K. and Frolich J. (1997) Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. Circulation **95:** 2068–2074
11 Massague J. (1990) The transforming growth factor-beta family. Annu. Rev. Cell. Biol. **6:** 597–641
12 Dijke P. T., Iwata K. K., Thorikay M., Schwedes J., Stewart A. and Pieler C. (1990) Molecular characterization of transforming growth factor type beta 3. Ann. NY Acad. Sci. **593:** 26–42
13 Zeiher A. M., Drexler H., Wollschlager H. and Just H. (1991) Modulation of coronary vasomotor tone in humans: progressive endothelial dysfunction with different early stages of coronary atherosclerosis. Circulation **83:** 391–401
14 Taddei S., Virdis A., Mattei P., Ghiadoni L., Gennari A., Fasolo B. et al. (1997) Hypertension causes premature aging of endothelial function in humans. Hypertension **29:** 736–743
15 Egashira K., Inoue T., Hirooka Y., Kai H., Sugimachi M., Suzuki S. et al. (1993) Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. Circulation **88:** 77–81
16 Parameswaran S. V., Steffens A. B., Hervey G. R. and de Ruiter L. (1977) Involvement of a humoral factor in regulation of body weight in parabiotic rats. Am. J. Physiol. **232:** R150–R157
17 Feld L. G., Van Liew J. B., Galaske R. G. and Boylan J. W. (1977) Selectivity of renal injury and proteinuria in the spontaneously hypertensive rat. Kidney Int. **120:** 332–343
18 Feld L. G., Cachero S., Van Liew J. B., Zamlauski-Tucker M. and Noble B. (1990) Enalapril and renal injury in spontaneously hypertensive rats. Hypertension **16:** 544–554
19 Shimamura T and Morrison A. B. (1975) A progressive glomerulosclerosis occurring in partial five-sixths nephrectomized rats. Am. J. Pathol. **79:** 95–106
20 Fukuzawa Y., Watanabe Y., Inaguma D. and Hotta N. (1996) Evaluation of glomerular lesion and abnormal urinary findings in OLETF rats resulting from a long-term diabetic state. J. Lab. Clin. Med. **128:** 568–578
21 Garcia de Boto M. J., Cobo A., Rodriguez J., Fernandez P., Rey C. and Santos F. (1996) Chronic renal failure and human growth hormone treatment do not modify endothelium-dependent reactions in the rat aorta in vitro. J. Auton. Pharmacol. **16:** 97–103
22 Teerlink J. R., Gray G. A., Clozel M. and Clozel J-P. (1994) Increased vascular responsiveness to norepinephrine in rats with heart failure is endothelium dependent: dissociation of basal and stimulated nitric oxide release. Circulation **89:** 393–401