Sinoatrial Node Dysfunction and Early Unexpected Death of Mice With a Defect of klotho Gene Expression

Kyosuke Takeshita, MD, PhD*; Toshihiko Fujimori, PhD*; Yoko Kurotaki; Haruo Honjo, MD, PhD; Hiroshi Tsujikawa, MD; Kenji Yasui, MD, PhD; Jong-Kook Lee, MD, PhD; Kaichiro Kamiya, MD, PhD; Kiyoyuki Kitaichi, PhD; Koji Yamamoto, MD, PhD; Masafumi Ito, MD, PhD; Takahisa Kondo, MD, PhD; Shigeo Iino, MD, PhD; Yasuya Inden, MD, PhD; Makoto Hirai, MD, PhD; Toyoaki Murohara, MD, PhD; Itsuo Kodama, MD, PhD; Yo-ichi Nabeshima, MD, PhD

Background—Homozygous mutant mice with a defect of klotho gene expression (kl/kl) show multiple age-related disorders and premature death from unknown causes.

Methods and Results—The kl/kl mice subjected to 20-hour restraint stress showed a high rate (20/30) of sudden death, which was associated with sinoatrial node dysfunction (conduction block or arrest). Heart rate and plasma norepinephrine of kl/kl mice, unlike those of wild-type (WT) mice, failed to increase during the stress. Intrinsic heart rate after pharmacological blockade of autonomic nerves in kl/kl mice was significantly lower than that in WT mice (380±33 versus 470±44 bpm; n=7). The sinus node recovery time after an overdrive pacing (600 bpm, 30 seconds) in kl/kl mice was significantly longer than in WT mice (392±37 versus 233±24 ms; n=6). In isolated sinoatrial node preparations, the positive chronotropic effect of isoproterenol was significantly less, whereas the negative chronotropic effect of acetylcholine was significantly greater in kl/kl than in WT mice. There was no degenerative structural change in the sinoatrial node of kl/kl mice. The precise localization of klotho was analyzed in newly prepared klotho-null mice with a reporter gene system (kl^geo). Homozygous kl^geo mice showed characteristic age-associated phenotypes that were almost identical to those of kl/kl mice. In the kl^geo mice, klotho expression was recognized exclusively in the sinoatrial node region in the heart in addition to parathyroid, kidney, and choroid plexus.

Conclusions—In the heart, klotho is expressed solely at the sinoatrial node. klotho gene expression is essential for the sinoatrial node to function as a dependable pacemaker under conditions of stress. (Circulation. 2004;109:1776-1782.)

Key Words: genetics ■ death, sudden ■ aging ■ sinoatrial node

A defect of klotho gene expression in mice results in a syndrome that resembles human aging.1 The mice are developmentally normal until the age of 3 weeks. Thereafter, they gradually become inactive and die prematurely at 8 to 9 weeks.1 The klotho mutant mouse (kl/kl), which carries deletion mutant allele disrupted by insertional transgene, is the first laboratory animal model for human aging caused by a single gene mutation.1 Despite extensive experimental studies to date, however, information available for understanding the pathophysiological role of klotho is still limited, and much remains to be clarified.

During breeding of the kl/kl mice, we found incidentally that the mice often died unexpectedly under a variety of stressful conditions such as transportation and high physical activity. Stress is known to contribute to exacerbation of cardiovascular dysfunction through a perturbation of the autonomic nervous system.2 In the present study, we investigated survival, autonomic reactions, and heart rate (HR) response of kl/kl mice subjected to a restraint stress to elucidate the exact causes of their premature death. The precise localization of klotho expression was analyzed in newly prepared klotho-null mice with a reporter gene system (kl^geo).

Methods

In Vivo Study of klotho Mutant Mice

All procedures were performed according to the protocol approved by the Animal Care and Use Committee of Nagoya University and

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From the Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya (K.T., T.K., S.I., Y.I., M.H., T.M.); Department of Pathology and Tumor Biology, Kyoto University Graduate School of Medicine, Kyoto (T.F., Y.K., H.T., Y.N.); Research Institute of Environmental Medicine and Department of Health Medicine, Nagoya University, Nagoya (H.H., K.Y., J.L., K. Kamiya, I.K.); Department of Molecular Medicine and Clinical Science, Nagoya University Graduate School of Medicine, Nagoya (K. Kitaichi); Division of Pathology, Clinical Laboratory, Nagoya University Hospital, Nagoya (M.I.); and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency (Y.K., Y.N.), Japan.

*K. Takeshita and T. Fujimori contributed equally to this work.
Correspondence to Yo-ichi Nabeshima, MD, PhD, Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe cho, Sakyo-ku, Kyoto 606-8501, Japan. E-mail nabemr@nmls.med.kyoto-u.ac.jp.

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that of Kyoto University. Homozygous klotho mutant (kl/kl) and wild-type (WT) mice were generated by crossing heterozygous klotho (kl/+H11001) mice.1 The genotypes were determined by Southern blot analysis and polymerase chain reaction. The male kl/kl and WT mice of littermates at 6 weeks were used for the present study because the typical phenotype of kl/kl mice is obvious at this age.1

RestRAINT stress was induced as described previously.3 The mice were individually housed in a room at 23°C on a 14/10-hour lighting schedule. Each mouse was acclimated for 1 week before initiation of restraint stress at 6 weeks. During the 20-hour restraint period (from noon on the first day to 8 AM on the second day), the mice were secured in well-ventilated, horizontal, 50-mL conical centrifuge tubes away from food and water.

ECG Monitoring and Catecholamine Measurements
ECGs were monitored by a telemetry system (TA10ETA-F20 [transmitter] and RMC-1 [receiver], Data Science).4 The transmitter was not implanted but was placed out of the body because of the small body size of kl/kl mice. The leads were fixed with minimal invasion. To examine autonomic influence on HR, cardiac muscarinic and β-adrenergic receptors were blocked by intraperitoneal administration of atropine (1 mg/kg) alone or together with propranolol (2 mg/kg).5 Plasma norepinephrine levels before and 2 and 20 hours after initiation of restraint stress were determined with reverse-phase liquid chromatography.

In Vivo Electrophysiological Study
The in vivo sinoatrial node function of mice under anesthesia was tested with a modified method of Berul et al.6 After thoracotomy, overdrive pacing was applied to the right atrial appendage at a cycle length of 100 ms for 30 seconds. The sinus node recovery time (SNRT) was defined as the interval between the last stimulus in the pacing train and the onset of the first spontaneous atrial excitation.6 The rate-corrected sinus node recovery time (CSNRT) was defined as SNRT minus steady state sinus cycle length.6

In Vitro Electrophysiological Study
The whole sinoatrial node and surrounding right atrial muscle were dissected from the mouse and superfused with Krebs-Ringer solution of the following composition (mmol/L): NaCl 120.3, KCl 4.0, CaCl2 1.2, MgSO 4 1.3, NaH 2 PO 4 1.2, NaHCO 3 25.2, and glucose 11.0. The solution was gassed with 95% O 2 /5% CO 2 to maintain pH at 7.4.7 Extracellular potentials were recorded at the periphery of the sinoatrial node with the use of a pair of modified bipolar electrodes.7 Acetylcholine (1 to 10 μmol/L) or isoproterenol (0.01 to 0.1 μmol/L) was added to the superfusate with cumulative increases of the concentration.

Targeted Disruption of klotho
Targeting vector was constructed as shown in Figure 1A. A genomic sequence8 of 8.2 kb for 5’ arm and 3.2 kb for 3’ arm was used in the targeting vector. We deleted 681 bp of the first exon,8 which includes the coding sequence corresponding to the first methionine and a part of the first β-glucosidase domain of klotho, from the genome and inserted coding sequence for LacZ-neomycin fusion protein (βgeo)9 and PGKneo cassette flanked with loxP sites. The targeting vector was transfected into CCE embryonic stem (ES) cells, and genetin-
resistant ES clones were screened by Southern blotting with the use of 5′/H11032 flanking sequence as a probe. Heterozygous fertilized eggs carrying the targeted allele were injected with the Cre recombinase expression vector and transferred into the oviducts of pseudopregnant females. H9252 H11002 transcribed under the control of the klotho promoter after PGKneo cassette is floxed out. The genomic DNA from the mice was digested with EcoRV and hybridized with neo probe. Animals without PGKneo cassette were selected (Figure 1B).

Histochemistry
For X-gal staining, tissues were dissected out and fixed with PBS containing 2% paraformaldehyde and 0.2% glutaraldehyde at 4°C. Signals were visualized after tissues were incubated in the X-gal reaction buffer at 37°C. Immunohistochemistry was performed with the anti-Klotho monoclonal antibody KM2119.10 For staining parathyroid hormone (PTH), anti-PTH rabbit anti-serum (Biogenesis) was used.

Statistical Analysis
The values presented are mean±SD unless otherwise specified. Statistical significance was evaluated by Student t test and ANOVA when appropriate. Cumulative surviving percentages of mice during the course of the restraint challenge were analyzed with a generalized Wilcoxon test. The in vitro electrophysiological study was analyzed with 2-factor ANOVA. A probability value <0.05 was considered significant.

Results
Restraint Stress Is Fatal for kl/kl Mice
During the 20-hour restraint challenge, survival rate of kl/kl mice decreased progressively, and 20 of 30 animals died by the end of the experiments (Figure 2A). In contrast, all 30 WT mice survived to the end of the restraint challenge. The difference in mortality between the 2 animal groups was significant (P<0.001). Autopsies of the 10 kl/kl mice immediately after the death revealed no intracranial hemorrhage, myocardial infarction, or cerebral infarction.

ECG monitoring was performed in 7 kl/kl and 7 WT mice during the restraint stress (Figure 2B). There were no differences in PQ, QRS, and QT intervals at baseline between kl/kl and WT mice (data not shown). In WT mice, RR interval was shortened during the stress by 27.3±3.4% at 4 hours from baseline (Figure 2C). In kl/kl mice, in contrast, RR interval was unchanged by 1 hour but then increased remarkably (by 70.1±8.9% at 4 hours from baseline). From 4 to 20 hours, 6...
The plasma norepinephrine levels at baseline in kl/kl mice (4.3
ng/mL) were approximately twice as high as those of WT mice (2.0
ng/mL) (Figure 2D). In WT mice, plasma norepinephrine was
dramatically increased during the stress and reached 18.7
ng/mL at 20 hours (P<0.05). In kl/kl mice, in contrast, plasma
norepinephrine was decreased slightly during the stress (7.00±1.2
ng/mL at 20 hours; P<0.05).

Sinoatrial Node Dysfunction in kl/kl Mice
Figure 3A shows the effects of pharmacological blockade of
autonomic influence on HR in 7 mice in vivo. Baseline RR
interval under anesthesia did not differ between kl/kl and WT
mice (106±19 versus 115±23 ms; P=NS). A single application of
atropine (1 mg/kg) resulted in no significant changes of RR interval from baseline in either kl/kl or WT
mice. Combined application of atropine (1 mg/kg) and
propranolol (2 mg/kg) resulted in a moderate prolongation of
RR interval in WT mice (by 21.7±4.8%; P<0.05), whereas
the prolongation was much greater in kl/kl mice (by
45.0±6.8%; P<0.001). The RR interval prolongation was the
result of sinus slowing but not of atrioventricular block. This
means a slower intrinsic HR in kl/kl mice.

Overdrive suppression of the sinoatrial node was tested in
6 WT and 6 kl/kl mice in vivo (Figure 3B). Spontaneous cycle
length of PP interval in kl/kl mice (228±45 ms) tended to be
longer than that in WT mice (183±23 ms), although the
difference did not reach statistical significance. The SNRT
after overdrive pacing in kl/kl mice was significantly longer
than that in WT mice (392±37 versus 233±24 ms; P<0.05).
The CSNRT in kl/kl mice was also significantly longer
than in WT mice (163±14 versus 52±5 ms; P<0.05).

Spontaneous activity of the sinoatrial node was also exam-
inated in isolated tissue preparations. All the preparations
showed regular spontaneous excitation under basal conditions
with cycle length (SPCL) at 169±21 ms (WT; n=7) and
173±21 ms for kl/kl mice (n=7). Application of isoprote-
nerol (0.01 to 0.1 μmol/L) resulted in a concentration-
dependent shortening of SPCL in both WT and kl/kl mice.
The SPCL shortening from baseline in kl/kl mice (by 8.0% to
19.4%) was significantly less than that in WT mice (by 18.7%
to 30.4%) (Figure 4A). Application of acetylcholine (1 to 10
μmol/L) resulted in a concentration-dependent prolongation
of SPCL. The SPCL prolongation by acetylcholine from
baseline in kl/kl mice (99.0% to 209.9%) was significantly
greater than that in WT mice (25.0% to 62.8%) (Figure 4B).
Pathological changes in the heart were examined in 3 kl/kl mice. Pacemaker cells in the sinoatrial node and their organization were morphologically normal, although the sinoatrial node artery showed mild to moderate Mönckeberg-type arteriosclerosis. There was no appreciable calcification of the conduction system. In addition, massive annular calcification was observed in the mitral and aortic valves.

**Generation of klotho-Null Mice With a LacZ Reporter Gene System**

To investigate the localization of klotho gene expression, we generated klotho-null mice with a reporter gene system (kl^−/−). Figure 1B shows Southern blotting of genomic DNA from mice. DNA was digested with EcoRV, and the blot was hybridized with 5′, 3′, or neo probe. The targeted allele (mu) and the β^neo allele (mu^′) were not discriminated by the blot with 5′ probe. The lower molecular size of the β^neo allele (without PGKneo cassette) was recognized by the blot with 3′ probe. In the blot with neo probe, 2 bands were detected for PGKneo and β^neo in targeted allele, whereas a single smaller band was detected in the β^neo allele. Thus, a targeted disruption of klotho gene was achieved in mice by insertion of β^neo.

The Klotho protein level in kidneys was examined by Western blots. The protein amount in the heterozygous kl^−/+ was approximately half that for WT mice, and the protein was undetectable in the homozygous kl^−/− (Figure 1C). This indicates that the deletion of exon 1 successfully resulted in the disruption of both KL1 and KL2 domains (Figure 1D). The age-associated phenotypes of the klotho-null mice were quite similar to those of original mutants (kl/kl). The klotho-null mice, like kl/kl mice, showed bradyarrhythmias in ECGs and a high incidence of sudden death under the restraint stress (data not shown).

**Klotho Expression in Mice**

Distribution of X-gal–positive cells in the heterozygous kidney was coincident with immunopositive cells for Klotho (Figure 5A, 5B, 5C). This provides an evidence for the transcription of β^neo and Klotho in the same manner. In addition to the kidney, strong X-gal staining was recognized in the parathyroid (Figure 5D) and the heart (Figure 5H, 5I, 5J, 5K) of heterozygous and homozygous animals. The signals in homozygote animals were stronger than that in heterozygote animals (Figure 5H, 5I). The signals in homozygote animals were stronger than that in heterozygote animals (Figure 5H, 5I). The parathyroid cells of heterozygotes were positively stained with anti-Klotho as well as with anti-PTH antibodies (Figure 5E, 5F). In the adult hearts of heterozygous (Figure 5H, 5J) and homozygous (Figure 5I, 5K) mutants, X-gal was positive in the subepicardial myocardial cells in the right atrium at the boundary with the superior vena cava, corresponding to the location of the sinoatrial node.

We also examined the expression of klotho during embryonic development (Figure 5L, 5M, 5N). X-gal signals were first recognized at 11.5 days post coitum (dpc) in the right atrium and sinus venosus of the heart, a part of the dorsal neural tube and the choroid plexus, and then in the parathyroid primordia at 12.5 dpc (Figure 5L). The signal became stronger up to 15.5 dpc (Figure 5M, 5N) and declined...
thereafter. At 15.5 dpc, broad areas in the right atrium and sinus venosus were positively stained with X-gal (Figure 5N). Precise localization of klotho around the sinoatrial node region was further examined in adult heterozygous kl\(^{+/−}\) mice by X-gal staining and immunolabeling of Cx40. It has been shown in an immunohistochemical study that the mouse sinoatrial node pacemaking cells are negative for Cx40 and Cx43 but positive for Cx45, unlike surrounding atrial cells (Cx40/43 positive; Cx45 negative).\(^{13}\) In the serial sections of kl\(^{−/−}\) mice, X-gal-positive cells were restricted in a region negative for Cx40 (Figure 6). This indicates specific expression of klotho in the sinoatrial node. Expression of klotho gene and Klotho protein in the sinoatrial node region was also confirmed by reverse transcription–polymerase chain reaction and Western blotting in small tissue specimens obtained from WT mice (data not shown).

**Discussion**

The present study first documented ECGs of kl/kl mice subjected to acute restraint stress during which two thirds of the animals died. Unlike WT mice, the HR of the kl/kl mice did not increase under the restraint stress; instead, most of the kl/kl mice showed bradyarrhythmias resulting from sinus arrest or sinoatrial block. In small-animal species, HR is kept at a very high level (400 to 500 bpm in mice at rest) to adapt to their relatively high metabolic rate.\(^{12}\) The failure of adjustment of HR to acute stress in kl/kl mice may therefore easily result in hemodynamic deterioration, culminating in unexpected premature death.

Plasma norepinephrine level at rest in kl/kl mice was significantly higher than that in WT mice. During the restraint stress, however, plasma norepinephrine of kl/kl mice was decreased, in contrast to its dramatic increase in WT mice. Such plasma norepinephrine behavior of kl/kl is in partial analogy with the effects of aging on the human sympathoadrenal system.\(^{13,14}\) This may be due in part to reduced neuronal reuptake of norepinephrine. Altered subcortical brain norepinephrine turnover in aged subjects may also be involved.\(^{14}\)

Our electrophysiological studies in vivo and in vitro have revealed significant sinus node dysfunction of kl/kl mice in terms of lower intrinsic HR, higher susceptibility to overdrive suppression, smaller positive chronotropic effect of isoproterenol, and greater negative chronotropic effect of acetylcholine. These observations bear certain similarities to sinus node dysfunction in humans at a higher incidence with senescence, although they are not identical.\(^{15}\) In our newly prepared klotho-null mice with a reporter gene system (kl\(^{−/−}\)), klotho gene expression has been recognized in the heart in addition to the parathyroid gland and the kidney. In the adult heart, X-gal was positive in the sinoatrial node region, which was confirmed by negative immunolabeling for Cx40. This provides evidence for specific expression of klotho in the sinoatrial node.

The exact molecular and cellular mechanisms underlying the sinoatrial node dysfunction in klotho-deficient mice remain unknown. Vascular insufficiency imposed by occlusion of the sinoatrial node artery via arteriosclerosis\(^{1}\) could induce functional abnormalities. However, this possibility is unlikely because only mild to moderate Mönckeberg-type arteriosclerosis was observed in the kl/kl mice, and no appreciable ischemic or degenerative changes were recognized in the microscopic architecture of the sinoatrial node.

Although early studies have suggested that the functions of the klotho gene product are partly mediated by a humoral factor,\(^{1,16}\) our recent findings suggest possibilities that Klotho may also act cell-autonomously in renal tubules and choroid plexus of the brain (A. Imura, MD, PhD and Y. Nabeshima,
MD, PhD, unpublished data, 2003). This may be the case in
the sinoatrial node. Expression of klotho in the sinoatrial node
cells may be essential in the function of certain ion channels
responsible for their pacemaking activity. More extensive
cellular electrophysiological studies will be required to eluci-
date this point. Prior experiments have demonstrated that
systemic delivery of klotho with the use of the recombinant
adenoviral vector can ameliorate various phenotypes in kl/kl
mice; those include an extension of life span, gain in body
weight, and restoration of gonadal cell formation and differ-
entiation.16 Such a procedure to rescue the pathological
abnormalities after onset of the phenotypes would provide
valuable information for understanding the mechanism un-
derlying the sinoatrial node dysfunction in klotho-deficient
animals. This will be an important subject of future studies.

Thus, the sinoatrial node may require klotho expression for
its normal and robust pacemaking activity in the heart under
a variety of pathophysiological conditions, although the exact
functional role of Klotho in the node remains to be elucidated.

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