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

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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...
that of Kyoto University. Homozygous klotho mutant (kl/kl) and wild-type (WT) mice were generated by crossing heterozygous klotho (kl/+) 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 Overdrive pacing was applied to the right atrial appendage at a cycle length of 100 ms to 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, MgSO4 1.3, NaH2PO4 1.2, NaHCO3 25.2, and glucose 11.0.7 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) and PGKneo cassette flanked with loxP sites. The targeting vector was transfected into CCE embryonic stem (ES) cells, and geneticin-
resistant ES clones were screened by Southern blotting with the use of $5^{\prime}$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. For staining para-thyroid 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
of the 7 kl/kl mice died. During the restraint stress ≥4 hours, ECGs of kl/kl mice showed bradyarrhythmias resulting from sinoatrial node dysfunction (sinoatrial conduction block or sinus arrest). The sinus slowing was followed by a slower junctional rhythm, culminating in complete asystole at the terminal phase (Figure 2B). The mice did not show supraventricular brady/tachyarrhythmias, atrial fibrillation, or ventricular brady/tachyarrhythmias, atrial fibrillation, or ventricular brady/tachyarrhythmias. There were no ST-T changes characteristic of myocardial ischemia.

The plasma norepinephrine levels at baseline in kl/kl mice (9.4±1.4 ng/mL) were approximately twice as high as those of WT mice (4.3±0.42 ng/mL) (Figure 2D). In WT mice, plasma norepinephrine was increased dramatically during the stress and reached 18.7±4.8 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 that in WT mice (163±14 versus 52±5 ms; P<0.05).

Spontaneous activity of the sinoatrial node was also examined in isolated tissue preparations. All the preparations showed regular spontaneous excitation under basal conditions. Cumulative application of Isp or ACh. A, Effects of Isp (0.01, 0.03, and 0.1 μmol/L). B, Effects of ACh (1, 3, and 10 μmol/L).
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<sup>−/−</sup>). 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<sup>geo</sup> allele (mu<sup>+</sup>) were not discriminated by the blot with 5′ probe. The lower molecular size of the β<sup>geo</sup> 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<sup>geo</sup> in targeted allele, whereas a single smaller band was detected in the β<sup>geo</sup> allele. Thus, a targeted disruption of klotho gene was achieved in mice by insertion of β<sup>geo</sup>. The Klotho protein level in kidneys was examined by Western blots. The protein amount in the heterozygous kl<sup>−/geo</sup> was approximately half that for WT mice, and the protein was undetectable in the homozygous kl<sup>−/−</sup> (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 bradyrhythmias 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<sup>geo</sup> 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 klotho-null mice, like kl/kl mice, showed bradyrhythmias in ECGs and a high incidence of sudden death under the restraint stress (data not shown).

![Figure 5. Expression of klotho in adult and embryo. A, B, Whole-mount and frozen sectioned kidneys of heterozygote were stained with X-gal. C, Serial section of B was immunolabeled for anti-Klotho antibody KM2119. Localization of signals was completely overlapped in a part of distal tubules (arrows). D, Parathyroids of heterozygote were positive for X-gal staining. Parathyroid cells were also positively stained with anti-PTH (E) and anti-Klotho (F) antibodies in serial frozen sections. Hearts of WT (G), heterozygote (H), and homozygote (I) stained with X-gal are shown. J, Section of heart shown in H. K, Close-up view of X-gal–positive cells shown in I. X-gal signals were localized at junction of right atrium and superior vena cava. L and M, Whole-mount X-gal staining of embryos at 12.5 dpc (L) and 15.5 dpc (M). N, Higher magnification of M. Strong signals were observed in parathyroid (p) and in right atrium (r.a.) of heart. Weak expression was observed in brain. Bars=500 μm in A, J, K, L, N; 50 μm in B and D; 1 mm in G and M.](http://circ.ahajournals.org/).
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 \textit{klotho} around the sinoatrial node region was further examined in adult heterozygous \textit{kl}<sup>-<sup>geo</sup></sup> 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). In the serial sections of \textit{kl}<sup>-<sup>geo</sup></sup> mice, X-gal–positive cells were restricted in a region negative for Cx40 (Figure 6). This indicates specific expression of \textit{klotho} in the sinoatrial node. Expression of \textit{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 \textit{kl/kl} mice subjected to acute restraint stress during which two thirds of the animals died. Unlike WT mice, the HR of the \textit{kl/kl} mice did not increase under the restraint stress; instead, most of the \textit{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. The failure of adjustment of HR to acute stress in \textit{kl/kl} mice may therefore easily result in hemodynamic deterioration, culminating in unexpected premature death.

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

Our electrophysiological studies in vivo and in vitro have revealed significant sinus node dysfunction of \textit{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. In our newly prepared \textit{klotho}-null mice with a reporter gene system (\textit{kl}<sup>-<sup>geo</sup></sup>), \textit{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 \textit{klotho} in the sinoatrial node.

The exact molecular and cellular mechanisms underlying the sinoatrial node dysfunction in \textit{klotho}-deficient mice remain unknown. Vascular insufficiency imposed by occlusion of the sinoatrial node artery via arteriosclerosis could induce functional abnormalities. However, this possibility is unlikely because only mild to moderate Mönckeberg-type arteriosclerosis was observed in the \textit{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 \textit{klotho} gene product are partly mediated by a humoral factor, 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, unpublished observations).
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 elucidate this point. Prior experiments have demonstrated that systemic delivery of klotho with the use of the recombinant adeno-viral 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 differentiation. Such a procedure to rescue the pathological abnormalities after onset of the phenotypes would provide valuable information for understanding the mechanism underlying 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.

References

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