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# Decreased expression of klotho gene in uremic atherosclerosis in apolipoprotein E-deficient mice

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ABSTRACT

Chronic renal failure (CRF) markedly accelerates the development of atherosclerosis, but the pathogenesis of uremic atherosclerosis remains to be elucidated. The klotho gene, predominantly expressed in the kidney, plays a key role in regulating aging and the development of age-related diseases in mammals. A loss of klotho results in multiple aging-like phenotypes including atherosclerosis. This study examines the relationship between the klotho expression and the development of accelerated atherosclerosis in uremic state.

Eight-week-old apolipoprotein E-deficient (apo-E$^{-/-}$) male mice underwent 5/6 partial kidney ablation to induce CRF or sham-operation. At 6 wk after nephrectomy, CRF mice showed significantly increased aortic plaque area fraction, aortic root plaque area and aortic cholesterol content as compared with non-CRF mice. Serum urea, total cholesterol and triglyceride concentrations were significantly higher in CRF apo-E$^{-/-}$ mice compared with non-CRF controls. Moreover, the expression of renal klotho gene and the serum levels of klotho protein were markedly decreased in CRF mice compared with controls.

These results suggested that CRF favored atherosclerosis in apo-E$^{-/-}$ mice and uremic atherosclerosis was accompanied by down-regulation of klotho expression.



## Introduction

Atherosclerosis is a generalized and inflammatory vascular disease which is extremely high in chronic renal failure (CRF) [1–3]. The morbidity and mortality from cardiovascular disease, especially atherosclerosis, is much higher in patients with CRF than in the general population [4]. Although a number of classic risk factors such as changes in plasma lipoproteins, BP, and plasma homocysteine have been documented to play predominant roles [5], the mechanism of increased cardiovascular disease in uremia remains to be elucidated. Uremia appears to have direct adverse effects on the arterial wall by favoring both arterial calcification and vascular disease in uremic patients.

The klotho gene, originally identified expressed in mice, suppresses the expression of multiple ageing-related phenotypes. The defect in klotho causes extensive age-related disorders including arteriosclerosis and calcification of soft tissues, resembling those in patients with premature ageing syndrome [6,7]. In vivo klotho gene delivery protects against endothelial dysfunction in rat with multiple risk factor syndrome and ameliorates renal damage [8]. Expression of renal klotho gene had been shown to be regulated in animal models and in humans with CRF [9,10]. Thus it is tempting to speculate that the propensity to accelerated lesion formation in uremia may involve attenuated expression of klotho. At present, however, little is known about the physiological relevance of regulation of the klotho expression and development of the atherosclerosis in uremic conditions.

To address the question of how uremia may accelerate atherosclerosis and how these complications affects klotho gene expression, we used apolipoprotein-E deficient (apo-E$^{-/-}$) mouse model, which was established to investigate the pathogenesis of uremic atherosclerosis [11,12], to study a possible acceleration of aortic atherosclerosis in CRF and the alteration of klotho gene expression in the atherosclerotic process.

## Materials and methods

*Animals and diet.* Male apo-E$^{-/-}$ mice (8-week-old, C57BL/6Jbom-Apoe$^{tm1Unc}$, backcrossed >10 generations onto the C57BL/6 background; Beijing Medical University) were kept (five mice per cage) on a 12-h light/dark cycle in a temperature-controlled room at 21–23 °C and given free access to water and regular laboratory chow.

All mice ($n$ = 70) were allocated to a western-type diet (10% wt/wt fat, 0.1% wt/wt cholesterol and 0.15% wt/wt cholalic acid) and

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maintained in SPF conditions according to the institute's guidelines. Food and water were provided ad libitum.

The experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

*Surgical procedures and experimental protocol.* Renal failure was induced by a two-step surgical procedure, as previously described [13]. We applied cortical electrocautery to the right kidney through a 2-cm flank incision and performed left total nephrectomy through a similar incision 2 weeks later. Control animals received sham-operation that included decapsulation of both kidneys. Special care was taken to avoid damage to the adrenals.

Blood samples of eight mice in either CRF group or control group were taken at the first surgery or at 2, 4, 6 weeks after the second intervention. Because the intensity of electrocoagulation determines the degree of CRF, we then aimed to produce a more moderate reduction in renal function and obtained serum urea concentrations in the CRF groups, which were more than 20 mM.

Following the completion of treatment mice were anesthetized and whole blood was collected. The kidneys were immediately dissected and preserved in 4% acid-formaldehyde solution.

*Serum biochemistry.* Blood was collected from the retrobulbar orbital plexus and subjected to centrifugation at 2000*g* for 10 min at 4 °C. And serum was stored at −20 °C. Serum levels of urea, total cholesterol and triglycerides were measured by a Hitachi Automatic analyzer 917 using reagents from Roche A/S (Hvidovre, Denmark). Serum urea is a more sensitive marker of uremia than plasma creatinine in mice, as the picric acid analysis (Jaffe's method) tends to overestimate creatinine levels due to the presence of interfering substances [14].

The level of serum klotho protein was determined with klotho ELISA kit.

*Quantification of aortic atherosclerotic lesions.* For evaluating the degree of atherosclerosis, the aortas were carefully freed of connective and adipose tissue before perfusion with 10% neutral formalin and embedded in paraffin. The aortic root was sectioned serially in 5-μm-thick slices from the cardiac end [15] and stained with hematoxylin–eosin. The cross-section surface area of the vessel and cross-section surface area of the lesion were measured with computer-assisted image analysis equipment from Olympus (BX50 light microscope, digital camera C-3030ZOOM, and DP-Soft Imaging System). Aortic root plaque area was expressed as the mean plaque area of the five sections. The extent of atherosclerosis was expressed as the percentage of surface area of the aorta covered by lesions [16].

The aortic lipids were extracted with chloroform/methanol [17]. After evaporation of the solvents under $N_2$, the lipids were redissolved in isopropanol with 1% Triton X100 (Sigma, St. Louis, MO), and cholesterol was quantified with an enzymatic method [18]. The results are reported as nmol cholesterol/cm$^2$ intimal surface area.

*RNA and cDNA preparation.* Total RNA was extracted from each individual mouse kidney using the TRIZOL reagent (Tiandz, China) and reverse-transcribed into cDNA. The resultant cDNA was amplified by SYBR Green1 fluorescence real-time RT-PCR. The PCR was directly monitored by the Bioer FQD-66A sequence detection system.

*Real-Time PCR.* In all experiments following isolation of total tissular RNA, klotho and β-actin mRNA levels were examined by real-time reverse transcription (RT) and polymerase chain reaction (PCR) by Light-Cycler (Roche Diagnostics, Basel, Switzerland).

The forward and reverse primer sequences for klotho were: 5′-AGTCTGGCATCTCTACAACAC-3′; reverse: 5′-GCAGAAGAGACGAG AGGTTG-3′ (amplifies a fragment of 217 bp). For mouse β-actin, forward: 5′-ATGCTCCCCGGGCTGTAT-3′; reverse: 5′-CATAGGAGTC CTTCTGACCCATTC-3′ (amplifies a fragment of 86 bp).

Each reaction mixture (20 μl) contained 1 μl of cDNA solution, 0.75 μl of each primer, 7.5 μl $H_2O$, and 10 μl of Light-Cycler Fast-start DNA Master SYBR Green I mix (Toyobo, Japan) which was detected at the end of each cycle to monitor the amount of PCR product formed during the cycle.

The real-time PCR protocol consisted of an initial step at 94 °C for 3 min followed by 40 cycles: 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. The fluorescence reading temperatures were 82–84 °C.

The temperature ramp was 20 °C/s. Standard curves were made by serial dilution of a pool of wild-type mouse heart cDNA. cDNA was run in duplicates or tetraplicates. To account for differences in cDNA preparation and cDNA amplification efficiency, the mRNA expression of each of the klotho was normalized by β-actin.

*Western blotting.* To measure the klotho protein level in the kidney, Western blotting was performed. Right kidney was excised and immediately frozen and stored in liquid nitrogen until protein extraction was performed. Kidneys were homogenized and lysed in lysis buffer and protein was isolated by homogenization of tissue sample in the lysis buffer containing protease inhibitors.

Equal amounts of protein were separated by SDS–PAGE (8% sodium dodecyl sulfate–polyacrylamide gel) and electrically transferred onto PVDF membranes by electroblotting for 2 h at 100v. The membrane was blocked in a 5% non-fat milk solution in TBS with 0.5% Tween-20.

The blot was incubated with 2 μg/ml of monoclonal anti-klotho antibodies (LS-C37134-50, anti-mouse IgG, LifeSpan Bioscience) for 60 min and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat Ig (1:5000, ZSGB-BIO) overnight.

The ECL Western blotting system (Bio-Rad, America) was used for detection. Bands were visualized, and band intensity was calculated with NIH image software and expressed as a percentage.

*Measurement of serum klotho concentration by ELISA.* Serum samples were transferred into the ELISA-plated containing enzyme-linked monoclonal antibody raised against klotho (sc-74205, anti-mouse klotho monoclonal antibody, Santa Cruz Biotechnology). After color development, absorbance at 450 nm was measured using a microplate reader (Tecan, Sunrise Fo 39300, Austria).

*Statistical analysis.* Data were analyzed by one-way ANOVA and unpaired *t* test with SPSS for Windows, version 13. Results were expressed as means ± SEM, with *n* indicating the number of mice studied. $P < 0.05$ was considered significant.

## Results

### *Effect of uremia on serum biochemistry*

Serum urea levels were used to assess renal function. In CRF mice, the serum urea concentration had a striking increase within 2 wk after 5/6 nephrectomy and remained elevated throughout the study. At 6 wk after surgical creation of CRF, the serum urea concentration increased by approximately 207% above baseline and tended to be stable, with minimal variations during the 6-wk period (Fig. S1A).

Meantime, the total serum cholesterol and triglyceride concentration were significantly increased in CRF mice compared with controls at 2 and 4 wk after induction of CRF (Fig. S1B and C). At 6 wk uremia, the total serum cholesterol concentration was 36% higher in CRF mice than in non-CRF mice (27.1 ± 0.8 vs. 19.9 ± 0.5 mmol/L; $P < 0.01$, Fig. S1B). And the serum triglyceride concentration of CRF mice at that time was increased by 69% above the levels of the control mice (4.9 ± 0.3 vs. 2.9 ± 0.2 mmol/L; $P < 0.01$, Fig. S1C).

*Effect of uremia on aortic atherosclerosis*

Before surgical creation of CRF, no lesion formation was observed neither macroscopically nor by sectioning the aortic roots from CRF mice or control mice. Also, no plaques were observed in aortic roots from 2 wk control mice. However, examination of the aortic root cross-sections from 2 wk CRF mice ($n = 6$) by light microscopy revealed lesions resembling early atherosclerotic plaques with accumulation of lipid-filled macrophages in the intima. At 4 and 6 wk after induction of uremia, the occupations of the aortic plaque in the vessels of CRF mice were much lager than that of control mice (Fig. 1).

At 6 wk after CRF, the total aortic plaque area fraction was increased 4.3-fold ($P < 0.001$) and the aortic cholesterol content was increased 4.1-fold ($P < 0.001$) in CRF mice compared with control mice (Fig. S2A and B). Meantime, the aortic root plaque area was increased by 59% ($P < 0.05$) in CRF mice compared with sham-operation mice (Fig. S2C).

*Effect of uremia on expression levels of klotho mRNA*

To determine mRNA expression levels in all apo-$E^{-/-}$ mice, we carried out the real-time PCR analysis which showed significant reduction of klotho mRNA expression in the kidneys of all apo-$E^{-/-}$ mice after induction of uremia or sham-operation (Fig. 2A).

On real-time PCR, the levels of klotho mRNA were demonstrated impairment in CRF mice compared with controls. At 2, 4, and 6 wk after the second surgery, the renal expression of klotho mRNA in CRF mice was 148 ± 25% ($P < 0.05$), 159 ± 15% ($P < 0.05$), and 227 ± 32% ($P < 0.05$) of that in control mice, respectively

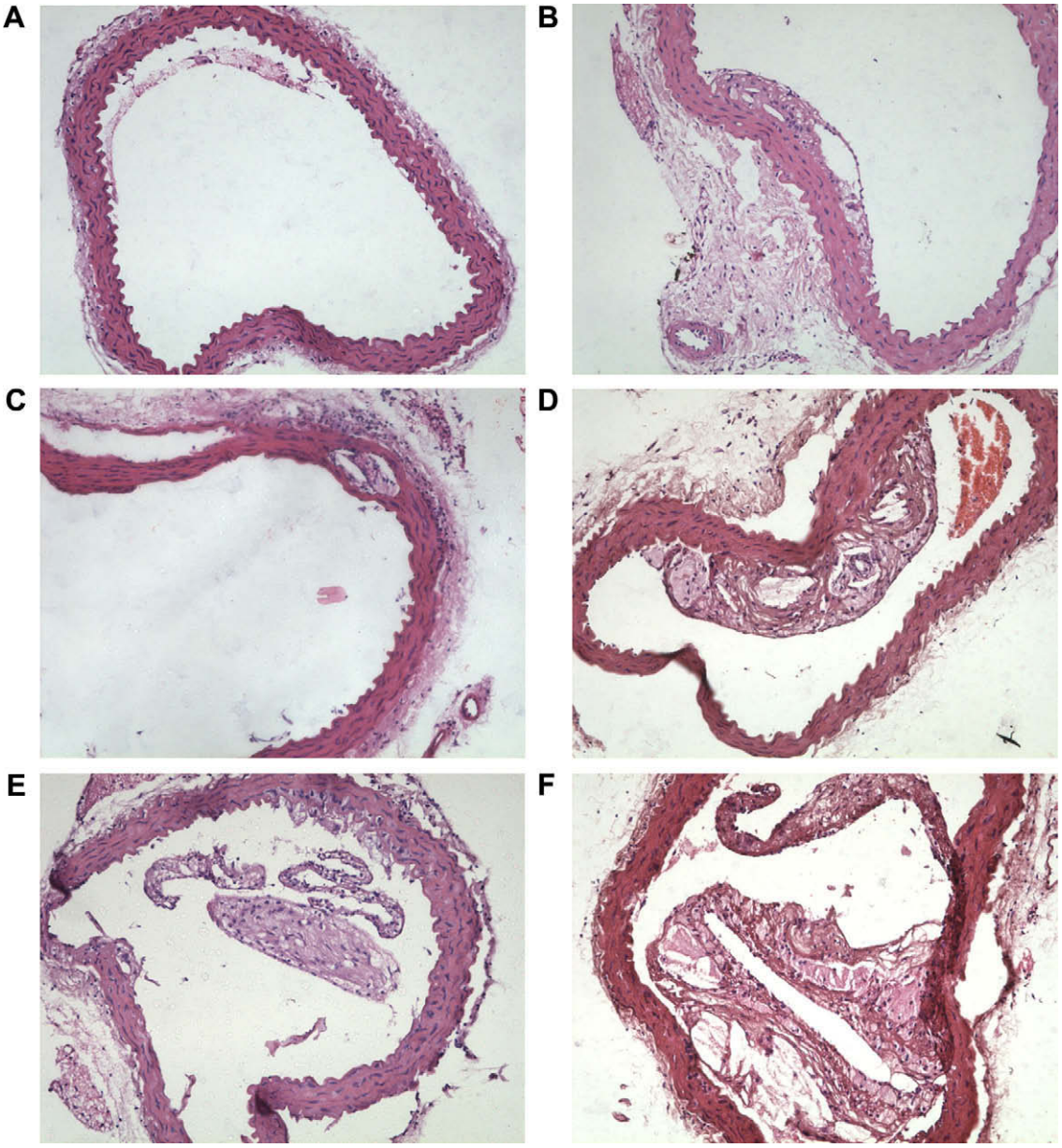


**Fig. 1.** Representative micrographs showing cross-sections of the aortic roots from control and CRF apo-$E^{-/-}$ mice. (A) Hematoxylin–eosin-stained cross-section of the aortic root from a control mouse at 2 wk after sham-operation and no plague formation could be demonstrated. (B) Aortic root cross-section from a CRF mouse at 2 wk after induction of uremia and early plaque formation. (C,E) Aortic root cross-sections from control mice at 4 and 6 wk after sham-operation. (D,F) Aortic root cross-sections from CRF mice at 4 and 6 wk after induction of uremia and advanced plaque formation. Original magnification, ×200.

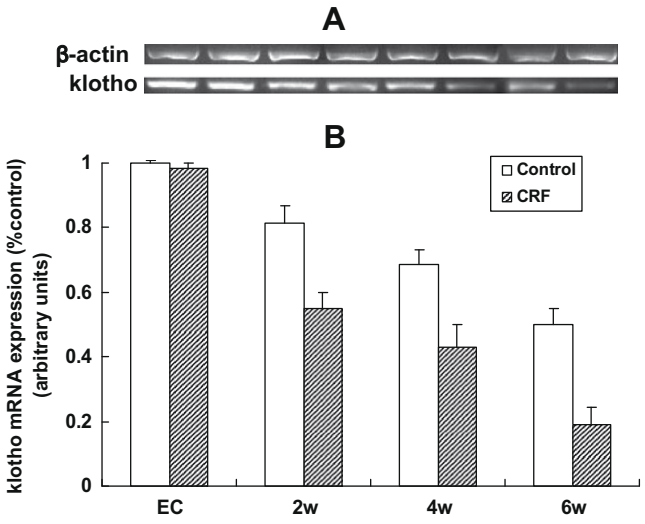


**Fig. 2.** Effect of uremia on expression of renal klotho mRNA in control and CRF apo-$E^{-/-}$ mice. Expression of klotho mRNA in kidney is significantly decreased in CRF mice as compared to control mice at 2, 4, and 6 wk after induction of uremia or sham-operation. The mRNA expression of renal klotho from each apo-$E^{-/-}$ mice was normalized by β-actin. Furthermore, the mRNA expression in CRF or control mice at 2, 4, and 6 wk were all normalized by the average of mRNA expression in control mice at EC, which was set at 1. $^{*}P < 0.05$ vs. control group, $n = 5$ in each group.

($n = 5$ in each group). Before 5/6 nephrectomy, the renal mRNA expression of klotho did not differ between kidneys from uremia and control mice (Fig. 2B).

*Effect of uremia on renal expression of klotho protein*

By Western blotting analysis using monoclonal antibody against mouse klotho, the klotho protein was detected at size of 130 kDa. Representative protein expression blotting was shown in Fig. 3A.

Relative expression in arbitrary units is shown in Fig. 3B, and protein expression of renal klotho was demonstrated reduction in all mice after surgery. Nevertheless, initially similar expression in CRF and control mice gradually differed after 5/6 nephrectomy.

Induction of uremia significantly promoted the reduction of klotho protein expression in CRF mice compared with control mice at 2, 4, and 6 wk after surgery (0.91 ± 0.03 vs. 0.64 ± 0.12 ($P < 0.01$), 0.68 ± 0.11 vs. 0.28 ± 0.23 ($P < 0.01$), and 0.60 ± 0.08 vs. 0.21 ± 0.19 ($P < 0.01$), respectively, $n = 5$ in each group).

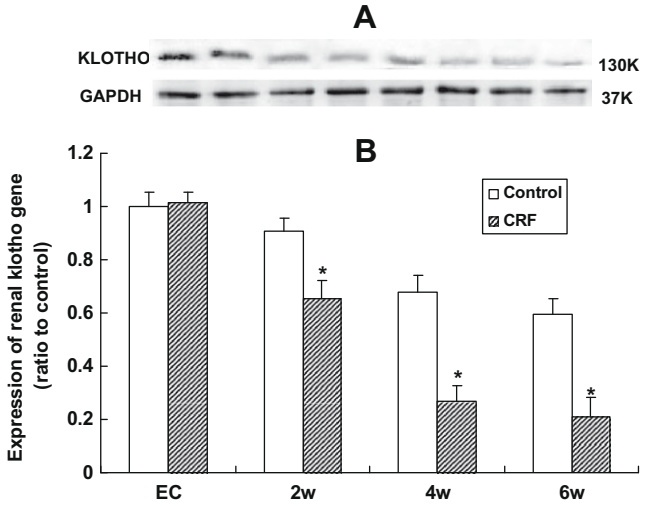


**Fig. 3.** Fold-changes in renal gene expression were determined with Western blotting in CRF mice versus control mice at EC or at 2, 4, 6 wk after surgery. $^{*}P < 0.05$ vs. control group, $n = 5$ in each group.

*Effect of uremia on serum level of klotho protein*

To address whether alterations in klotho mRNA levels were translated into those in protein expression levels, klotho protein concentration in serum was measured.

As shown in Fig. 4, klotho protein levels in serum significantly decreased in CRF mice compared with control mice at 2, 4, and 6 wk after surgery (1727 ± 79 vs. 1377 ± 86 ($P < 0.05$), 1554 ± 92 vs. 1027 ± 108 ($P < 0.05$), and 1527 ± 87 vs. 537 ± 103 pg/ml ($P < 0.05$), respectively, $n = 5$ in each group). These results not only demonstrated that the soluble klotho protein existed in extracellular fluids in mice, but shown that the klotho protein levels in serum were suppressed at the uremic condition.

## Discussion

Atherosclerosis integrates the response to a number of insults, and consequently, the accelerated atherosclerosis found in CKD patients is associated with activation of a variety of humoral and tissue mechanisms. Although many risk factors for atherosclerosis are identified to be involved in uremic patients, the aspects of the uremic milieu that are responsible for the accelerated atherosclerosis remain incompletely understood.

Recently, the klotho gene, which plays a pivotal role in regulating aging and the development of age-related diseases in mammals, alluring more focus. A loss of klotho results in multiple aging-like phenotypes, such as arteriosclerosis, skin atrophy, osteoporosis, vascular calcifications and infertility [6,7]. In vivo klotho gene delivery using an adenoviral expression vector increases endothelium-dependent NO synthesis in a rat model of multiple atherogenic risk factors [8]. Oxidative stress is often suggested to play central roles in endothelial dysfunction and atherosclerosis [19]. It is interesting to note that klotho protein itself or its metabolites may function as a humoral factor and protect the vascular endothelial cells which are continuously exposed to oxidative stress [20,21]. And in humans, a decrease of klotho expression was observed in chronic renal diseases [10]. We think that the alteration of klotho expression might participate in accelerating the atherosclerosis in uremia. Therefore, we studied the klotho expression in partial nephrectomy and sham-operation apo-$E^{-/-}$ mice to gain new insight into specific effects of putative uremia on the development of atherosclerosis.

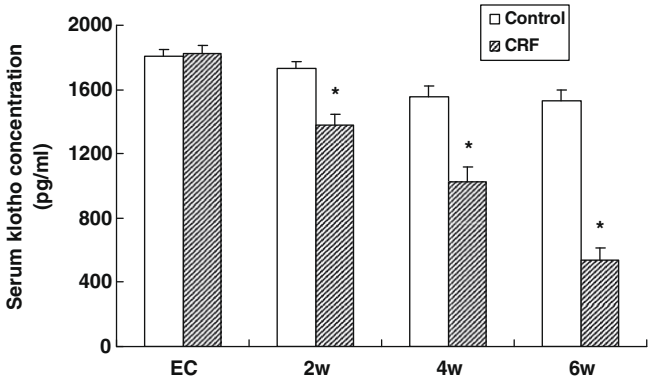


**Fig. 4.** Serum klotho concentration were detected in CRF and control mice at EC or at 2, 4, 6 wk after surgery. $^{*}P < 0.05$ vs. control group, $n = 5$ in each group.

In the present study, uremia was induced in apo-E$^{-/-}$ mice to investigate the role of klotho gene in accelerated atherosclerosis. The apo-E$^{-/-}$ mouse, whose predisposition was liable to form human-like atherosclerotic lesions and its combined susceptibility to development of uremia upon partial nephrectomy, provides an excellent model for studying uremic atherosclerosis [22].

In our research, we found that the development of aortic plaque was enhanced in 5/6 nephrectomy apo-E$^{-/-}$ mice compared with sham-operation controls. Moreover, 6 wk after induction of renal failure, the total aortic plaque area fraction and aortic cholesterol accumulation were significantly increased in CRF mice. The results were in agreement with previous reports [13,23]. Moreover, the total serum cholesterol and triglyceride concentration were higher in CRF mice than in control mice after induction of uremia.

Increasing evidence suggests that oxidative stress within the arterial wall may be important for progression of atherosclerotic lesions in uremia. The abnormal function and catabolism of lipoproteins as a result of oxidation or glycation are characteristics of uremia that may further enhance the atherogenicity of plasma lipoproteins [24,25]. Hence, the higher level of lipoproteins might contribute to the accelerated formation of atherosclerosis in uremic mice. The regulation of lipoproteins through klotho cannot be excluded in the mechanisms of abnormal function and catabolism of lipoproteins in uremia. This idea was strengthened by the findings of Noriko et al. indicating that statin treatment significantly improved arteriosclerotic lesions and vascular damage in rats via enhancing anti-aging klotho protein expression [26]. But it needs further investigations to exhibit the relation between klotho and catabolism of lipoproteins.

In humans, klotho gene allele is related to an early-onset occult coronary artery disease that is independent of other coronary risk factors [27]. Because of sustained circulatory and/or oxidant stress, the klotho expression was reported to decrease in 5/6 nephrectomized rats [28]. And to data, overexpression of the klotho gene in mice suppresses aging and extends lifespan which may involve the mechanism of suppression of insulin signaling and oxidant stress [20]. However, there is no well-documented evidence for a direct relationship between attenuated klotho expression and alterations in uremic atherosclerosis.

It was demonstrated that klotho mRNA and protein are predominantly expressed in the kidney [6]. In our study, renal klotho expression was strikingly reduced at both mRNA and protein levels in CRF mice. Furthermore, the levels of renal klotho expression in CRF mice showed significantly lower than the control mice after induction of uremia or sham-operation. The changes in klotho gene expression were accompanied by progressive renal failure and aggravation of plaque progression in the aorta of uremic apo-E$^{-/-}$ mice. Meantime, the levels of serum klotho protein were decreased in CRF mice compared with control mice. Because many tissues do not inherently express the klotho gene, the disruption of renal klotho gene expression may result in systemic disorders such as arteriosclerosis [6,7]. The present research suggested that uremia may be promoted the proceeding of atherosclerosis via impairment in klotho expression and enhancement of the oxidative stress in CRF. Also, the mechanisms by which klotho gene might modify oxidative stress remain to be defined.

In summary, the present investigation has shown that uremia markedly accelerates atherogenesis in apo-E$^{-/-}$ mice. And this proceeding is accompanied by down-regulation of anti-aging klotho expression. A novel idea may be provided that decreased klotho expression may be an important impetus for accelerated atherogenesis in uremic apo-E$^{-/-}$ mice. And if so, although further investigation is required, anti-aging klotho gene would be a promising potential therapeutic factor for use in this disease, especially in an elderly population.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.046.

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