Abstract—The klotho gene, originally identified by insertional mutagenesis in mice, suppresses the expression of multiple aging-associated phenotypes. This gene is predominantly expressed in the kidney. Recent studies have shown that expression of renal klotho gene is regulated in animal models of metabolic diseases and in humans with chronic renal failure. However, little is known about the mechanisms and the physiological relevance of the regulation of the expression of the klotho gene in the kidney in some diseased conditions. In the present study, we first investigated the role of angiotensin II in the regulation of renal klotho gene expression. Long-term infusion of angiotensin II downregulated renal klotho gene expression at both the mRNA and protein levels. This angiotensin II–induced renal klotho downregulation was an angiotensin type 1 receptor–dependent but pressor-independent event. Adenovirus harboring mouse klotho gene (ad-klotho, 3.3×10^10 plaque forming units) was also intravenously administered immediately before starting angiotensin II infusion in some rats. This resulted in a robust induction of Klotho protein in the liver at day 4, which was still detectable 14 days after the gene transfer. Ad-klotho gene transfer, but not ad-lacZ gene transfer, caused an improvement of creatinine clearance, decrease in urinary protein excretion, and amelioration of histologically demonstrated tubulointerstitial damage induced by angiotensin II administration. Our data suggest that downregulation of the renal klotho gene may have an aggravative role in the development of renal damage induced by angiotensin II, and that induction of the klotho gene may have therapeutic possibilities in treating angiotensin II–induced end organ damage. (Hypertension. 2002;39:838-843.)

Key Words: aging ■ angiotensin II ■ gene regulation ■ renal disease ■ hypertension, experimental

The klotho gene, identified by insertional mutagenesis in mice, is a suppressor of the expression of multiple aging phenotypes similar to age-related diseases in humans, such as arteriosclerosis, osteoporosis, infertility, pulmonary emphysema, and short lifespan.1 Interestingly, expression of klotho mRNA in the kidney can be only faintly detected in the prenatal rat, and it is markedly augmented after 4 days of age.2 Although the klotho gene has a role in phenotypic alterations in various organs, expression of klotho mRNA is predominantly observed in the kidney,1 suggesting that the Klotho protein or its metabolites may function as humoral factors. Recent studies have shown that expression of renal klotho gene is regulated in animals3 and in humans4 in some diseased conditions. At present, however, the mechanism regulating klotho gene expression is poorly understood.

In the present study, we have investigated the role of angiotensin (Ang) II in the regulation of renal klotho gene expression. In addition, to clarify the possible physiological role of the klotho gene in the Ang II–infused rats, exogenous klotho gene was delivered into Ang II–infused rats, and functional and histological changes in the kidney were analyzed.

Methods

Animal Models

The experiments were performed in accordance with the guidelines and practices established by the Animal Center for Biomedical Research, University of Tokyo, Faculty of Medicine. The rat Ang II hypertension model was induced in male Sprague-Dawley rats (Nippon Bio-Supply Center, Tokyo, Japan) by the continuous infusion of [Val^2]-Ang II (Sigma) at a dose of 0.7 mg/kg per day via an osmotic minipump (Alza) as described previously.4 In some experiments, the selective angiotensin type 1 receptor antagonist losartan (25 mg/kg per day; a gift from Merck, Rahway, NJ) or the nonspecific vasodilator hydralazine (15 mg/kg per day; Sigma) was given in the drinking water, beginning 2 days before pump implantation and continuing throughout Ang II infusion. Norepinephrine (NE) was infused at a dose of 2.8 mg/kg per day. Hemodynamic variables were measured by tail-cuff plethysmography (UR-5000; Ueda Seisakusyo).
RNA Isolation and Northern Blot Analysis

After total RNA was obtained using Isogen (WAKO), mRNA was subsequently isolated using oligotex-dT30 (Roche Diagnostics). Mouse klotho cDNA was labeled with [α-32P] dCTP (DuPont NEN) using commercial kits (Nippon Gene). Hybridized bands were visualized and quantified using a bio-imaging analyzer (BAS 2000; Fuji Photo Film), and band density was normalized to the intensity of band of GAPDH mRNA.

In Situ Hybridization

In situ hybridization was performed as previously described with minor modifications. Briefly, RNA probes corresponding to the sense and antisense strands of the klotho cDNA were prepared using the MAXI script kit (Ambion) with fluorescein (FITC)-12-UTP (ChromaTide; Molecular Probes). The sections were treated with RNase, blocked with 10% normal sheep serum, and hybridized for 30 minutes at room temperature with sheep polyclonal anti-FITC (ChromaTide; Molecular Probes). The sections were treated with RNase, blocked with 10% normal sheep serum, and hybridized for 30 minutes at room temperature with sheep polyclonal anti-FITC (F(ab')2) fragments conjugated with alkaline phosphatase (Dako). Color development was performed in the nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate solution overnight in the dark.

Protein Purification and Western Blot Analysis

Protein was isolated as described previously. Antibody against mouse Klotho (a kind gift from Kyowa Hakko, Kogyo, Shizuoka, Japan) was used at a 1:4000 dilution. The ECL Western blotting system (Amersham Life Sciences) was used for detection. Bands were visualized, and band intensity was calculated as described previously.

Construction of Recombinant Adenoviruses and Adenovirus-Mediated Gene Transfer

Adenovirus encoding the mouse klotho gene (a kind gift from Kyowa Hakko) was constructed by the cosmid cassettes and adenovirus DNA-terminal protein complex (COS-TPC) method as described elsewhere. Adenovirus harboring the Escherichia coliβ-galactosidase gene was designated as ad-lacZ. Purified recombinant adenovirus was injected to rats through the tail vein at a dose of 3×1010 plaque forming units (pfu) on the first day of Ang II infusion.

Morphological Analysis

Sections (3 µm in thickness) of paraffin-embedded tissue specimens were stained with the periodic acid–Schiff reagent and counterstained with hematoxylin. Tubulointerstitial injury was defined as tubular dilation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, and thickening of the tubular basement membrane and was quantified on a scale of 0 to 4, as described previously.

Statistical Analysis

Data are expressed as mean±SEM. ANOVA followed by a multiple comparison test was used for comparisons of the initial data before expression as a percentage of the control. A value of P<0.05 was considered statistically significant.

Results

Ang II Downregulates Renal Expression of klotho mRNA and Klotho Protein

Continuous administration of Ang II markedly downregulated renal expression of klotho mRNA levels in a time-dependent manner (Figure 1A and 1B). In situ hybridization revealed that klotho mRNA was predominantly expressed in the renal tubular epithelial cells, and that Ang II administration apparently reduced klotho mRNA expression in these regions (Figure 1C). Immunoblot analysis using monoclonal antibody against human Klotho detected 2 bands at sizes of ~130 kDa, which was significantly downregulated in response to Ang II administration (Figure 1D and 1E).

Pressor-Dependency of Ang II–Induced Renal klotho mRNA Downregulation

We then tested the effects of antihypertensive agents on Ang II–induced klotho mRNA downregulation. Losartan, but not hydralazine, completely blocked the Ang II–induced klotho mRNA downregulation (Figure 2A and 2B), which suggested that Ang II–induced klotho mRNA downregulation is a pressor-independent event. Long-term administration of NE, which resulted in comparable hypertensive effect as that of Ang II, did not affect renal klotho mRNA expression (Figure 2C and 2D). Continuous infusion of a lower dose of Ang II (0.25 mg/kg per day), which did not affect systolic blood pressure and thus was considered a nonpressor dose, downregulated klotho mRNA expression in the kidney (Figure 2C and 2D). These results support a crucial role of Ang II in the downregulation of klotho mRNA expression in the kidney.
Klotho Protein Expression After In Vivo klotho Gene Transfer

To investigate the possible physiological role(s) of klotho gene expression in the kidney, we attempted in vivo gene transfer of klotho gene in the Ang II–infused rat. Rats were given either ad-klotho or ad-lacZ at a dose of 3.3 × 10^9 pfu via the tail vein on the first day of continuous administration of Ang II. Klotho protein expression in various tissues was examined 4 days after gene transfer. In the liver (Figure 3A), aorta, and heart (Figure 3B), no immunodetectable band was observed in control rats or rats given ad-lacZ plus Ang II. In contrast, 4 days after ad-klotho administration, robust Klotho protein expression was observed in the liver. There were at least 3 immunodetectable bands in the liver after ad-klotho infection (Figure 3A). In the aorta and heart, protein expression of exogenous klotho gene was also demonstrated 4 days after ad-klotho administration (Figure 3B). Expression of Klotho protein was still detectable in the liver 14 days after the gene transfer (Figure 3C).

Effect of klotho Gene Transfer on the Ang II–Induced Renal Dysfunction

We have shown previously that continuous Ang II administration into rats for 7 days increased urinary protein excretion and decreased creatinine clearance, a marker for glomerular filtration rate. Thus, in the present study, we investigated whether in vivo klotho gene transfer has any effects on the renal damage induced by Ang II. Ad-klotho gene transfer significantly reduced the Ang II–induced increase in urinary protein excretion at both day 7 and day 14 after Ang II administration. Although ad-klotho gene transfer did not significantly improved creatinine clearance 7 days after Ang II infusion, it did partially suppress the Ang II–induced decrease in creatinine clearance 14 days after Ang II administration. No significant effects of ad-lacZ gene transfer could be observed in the Ang II–induced increase in the urinary protein excretion or decrease in creatinine clearance (Table).

Effect of klotho Gene Transfer on Morphological Changes of the Kidney After Ang II Administration

Finally, we examined whether in vivo klotho gene transfer could affect morphological kidney damage induced by 14 days of Ang II infusion in the tubulointerstitial regions. In the kidney of rats given Ang II plus ad-lacZ, marked tubulointerstitial damage (ie, dilated tubules, tubular cast formation, sloughing of epithelial cells, infiltration of inflammatory cells) and thickening of the vascular wall could be observed (Figure 4A through 4E). These changes appeared to be reduced in the kidneys of rats given Ang II plus ad-klotho (Figure 4F through 4I). Scores of tubulointerstitial damage (TI score) in the control rats, rats given Ang II administration alone, and rats given Ang II plus ad-lacZ were 0.0±0.0
Body Weights, Hemodynamic Variables, and Renal Function in the Ang II–Infused Rat With or Without Adenovirus-Mediated Gene Transfer

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Pressure, mm Hg</th>
<th>Heart Rate, ppm</th>
<th>Body Weight, g</th>
<th>Proteinuria, mg/day</th>
<th>Creatinine Clearance, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=9)</td>
<td>133±3</td>
<td>354±6</td>
<td>292±2</td>
<td>9.7±1.3</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>7 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG II alone (n=9)</td>
<td>197±14†</td>
<td>415±16†</td>
<td>224±8†</td>
<td>28.2±7.2*</td>
<td>1.2±0.2†</td>
</tr>
<tr>
<td>ANG II+ad-klotho (n=10)</td>
<td>196±14†</td>
<td>427±22†</td>
<td>220±8†</td>
<td>10.4±2.5§</td>
<td>1.3±0.1†</td>
</tr>
<tr>
<td>ANG II+ad-lacZ (n=10)</td>
<td>193±7†</td>
<td>436±13†</td>
<td>223±7†</td>
<td>22.3±6.0*</td>
<td>1.2±0.1†</td>
</tr>
<tr>
<td>14 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG II alone (n=7)</td>
<td>199±12†</td>
<td>441±12†</td>
<td>200±13†</td>
<td>46.8±14.0*</td>
<td>0.9±0.1†</td>
</tr>
<tr>
<td>ANG II+ad-klotho (n=7)</td>
<td>205±7†</td>
<td>441±30†</td>
<td>205±7†</td>
<td>23.3±6.5§</td>
<td>1.3±0.1§</td>
</tr>
<tr>
<td>ANG II+ad-lacZ (n=9)</td>
<td>199±6†</td>
<td>429±20†</td>
<td>202±7†</td>
<td>42.9±7.6*</td>
<td>0.9±0.1†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05 and †P<0.01 vs control (ie, untreated) rats, respectively. ‡P<0.05 and §P<0.01 vs rats given ad-lacZ plus Ang II for the same administration duration.

(6), 2.2±0.1 (n=10, P<0.001 versus control), and 2.1±0.1 (n=18) (P<0.001 versus control and P=NS versus Ang II alone), respectively. TI score in the rats given Ang II plus ad-klotho was 1.3±0.1 (n=18), which was significantly smaller compared with that of rats given Ang II alone (P<0.001) or rats given Ang II plus ad-lacZ (P<0.01). These findings suggested that ad-klotho gene transfer ameliorated morphological kidney damage induced by long-term continuous administration of Ang II.

Discussion

In the present study, we showed 2 major findings. First, continuous administration of Ang II reduced renal klotho gene expression. Second, adenovirus-mediated klotho gene transfer to rats ameliorated Ang II–induced functional and morphological damages in the kidney. These data suggest a possible role of downregulation of the klotho gene in the development of Ang II–induced renal damage in rats.

In situ hybridization revealed that klotho mRNA was predominantly expressed in the renal tubular epithelial cells. This distribution was similar to findings in both the human and the mouse.9 Northern blot analysis showed that Ang II administration markedly downregulated klotho mRNA expression, which was consistent with the findings of in situ hybridization. We found that losartan, but not hydralazine, inhibited Ang II–induced renal klotho mRNA downregulation. In addition, NE did not downregulated renal klotho expression, and subpressor doses of Ang II, although to a lesser extent, decreased renal klotho mRNA expression. These findings suggest that Ang II, but not hypertension per se, plays a pivotal role in renal klotho gene expression.

In previous studies, downregulation of klotho mRNA in the kidney has been reported in other animal hypertension models, such as the spontaneously hypertensive rat and deoxy-
corticosterone acetate (DOCA)-salt hypertensive rat. Levels of renin and Ang II may be normal to suppressed in these animal models, which may indicate the presence of a mechanism other than activation of the renin-angiotensin system for renal klotho mRNA downregulation. However, downregulation of renal klotho mRNA was observed in spontaneously hypertensive rats at 60 weeks of age and in DOCA-salt hypertensive rats at 6 weeks after the initial operation. Activity and/or concentration of plasma renin may increase at these time points in these models. Therefore, the possibility of the involvement of activation of the renin angiotensin system in the renal klotho downregulation in these hypertensive models seems to still remain. In the present study, we did not examine whether klotho mRNA downregulation was a consequence of the direct action of Ang II on these cells or of some other humoral factors modulated by the Ang II–angiotensin type 1 receptor axis. To answer this question, we used established cell line of cultured rat proximal tubular epithelial cells. However, expression of the klotho gene, at either the mRNA or protein level, could not be observed in these cell lines (data not shown). Therefore, in vivo systems should be used to answer this question in future studies.

To investigate the possible physiological relevance of the klotho gene on the renal function of rats with continuous Ang II infusion, klotho gene was delivered to Ang II–infused rats by means of an adenovirus vector. When ad-klotho was intravenously administered, increases in Klotho protein expression could be observed in the liver, heart, and aorta. However, no apparent difference in the Klotho protein expression was observed in the kidney after ad-klotho transfer. There were at least 3 immunodetectable bands in the liver after ad-klotho gene transfer, the sizes of which were equal to or smaller than endogenous Klotho protein expressed in the kidney. In addition, a band whose size was greater than the endogenous renal Klotho protein was detected in the aorta and heart. These findings suggest that Klotho protein may undergo posttranslational modification and/or degradation in these organs and should be a topic of future studies. Western blot analysis revealed that Klotho protein expression was still observed in the liver 14 days after the exogenous klotho gene transfer. We found that gene transfer of ad-klotho, but not of ad-lacZ, ameliorated Ang II–induced renal dysfunction, ie, significantly suppressed the Ang II–induced decrease in creatinine clearance and increase in urinary protein excretion. In addition, histological examination revealed that ad-klotho gene transfer significantly ameliorated Ang II–induced morphological damage. These findings indicate that induction of the klotho gene might protect against Ang II–induced renal damage. Conversely, Ang II–induced klotho mRNA downregulation in the kidney may aggravate renal damage induced by this octapeptide.

In the present study, klotho gene delivery was performed via tail vein application, and overexpression of Klotho protein could not be observed in the kidney (Figure 3B). One may question why klotho overexpression in nonrenal tissues can ameliorate Ang II–induced damage in the kidney. Shirakaki-Lida et al. have recently reported a similar observation that intravenous administration of ad-klotho construct resulted in increased klotho mRNA expression in the liver but not in other organs, including brain, spleen, and kidney. Nevertheless, they found that restricted expression of the klotho gene in the liver could compensate for the function of Klotho protein expressed in the kidney in these genetically altered mice. Our data further support the possibility that Klotho protein secreted by proteolytic cleavage of the membrane form, its metabolites, or downstream signaling molecule(s) may possibly function as humoral factors. The notion that some humoral factors may mediate the functions of klotho may be further supported by a previous report that parabiosis between wild-type and heterozygous klotho mice resulted in restoration of endothelial function in heterozygous klotho mice.

Our data suggested that downregulation of renal klotho gene expression may play a role in exacerbating Ang II–induced renal injury and that induction of klotho gene expression can possibly be a useful strategy in ameliorating Ang II–induced end organ damage.

Continuous administration of Ang II, but not NE, decreased klotho mRNA expression in the rat kidney. This was a pressor-independent event, indicating the crucial role of Ang II in downregulating klotho gene expression in the kidney. In addition, we showed that adenovirus mediated exogenous klotho gene transfer ameliorated renal damage induced by Ang II. Our data suggested a novel possibility that renin angiotensin system is involved, or at least modulates, the aging-related organ damage. This hypothesis should be investigated in future studies.

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References


In Vivo klotho Gene Transfer Ameliorates Angiotensin II-Induced Renal Damage
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