Iron chelation and a free radical scavenger suppress angiotensin II-induced downregulation of *klotho*, an anti-aging gene, in rat

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Abstract Administration of angiotensin II to rats decreases renal expression of *klotho*, an aging-related gene, and also causes abnormal iron deposition in renal cells. Here we have examined the effects of iron overload and iron chelation on renal expression of *klotho* in untreated rats and rats treated with angiotensin II. Administration of iron–dextran caused a downregulation of *klotho* expression, and iron chelation suppressed the angiotensin II-induced downregulation of this gene. In addition, a free radical scavenger (T-0970), which effectively decreased plasma levels of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$), suppressed angiotensin II-induced downregulation of *klotho*. Collectively, these findings suggest that abnormal iron metabolism and increased oxidative stress are involved in the mechanism of angiotensin II-mediated modulation of *klotho* expression.

Key words: Angiotensin II; Aging; Iron metabolism; Kidney; Oxidative stress

1. Introduction

The murine *klotho* gene was identified as a suppressor of multiple aging phenotypes similar to age-related diseases in humans, such as atherosclerosis, cognition impairment, emphysema, and short lifespan [1–3]. Interestingly, despite the fact that various actions of the *klotho* gene have been suggested from mouse models of *klotho* deficiency, this gene is expressed almost exclusively in the renal tubular cells. Recent studies have suggested that a human functional variant of *klotho* is associated with both reduced human longevity [4] and an occult coronary artery disease that is independent of other confounding factors [5], which may indicate that *klotho* gene expression also has a role in the development of the aging-related phenotypes in humans. Renal expression of *klotho* gene is downregulated in patients with severe renal dysfunction [6] and in animals in response to sustained circulatory stress [7] and metabolic disorders [8]; however, the mechanism underlying the modulation of *klotho* expression in vivo still remains largely unclear.

We have recently shown that continuous administration of angiotensin II, but not catecholamines, decreases renal *klotho* expression in rats, which may in turn have a role in the augmentation of angiotensin II-induced renal injury [9]. In another set of experiments, we found that administration of angiotensin II to rats causes apparent deposition of iron in the kidney [10]. In the present study, we have investigated whether iron chelation can inhibit angiotensin II-induced downregulation of renal *klotho* expression, and whether iron overload can decrease *klotho* expression.

2. Materials and methods

2.1. Animal models

Angiotensin II was continuously infused into male Sprague–Dawley rats by subcutaneous implantation of an osmotic minipump (Alzet model 2001; Alza Pharmaceutical) as described previously [11]. Briefly, Val^5-angiotensin II (Sigma) was infused at a dose of 0.7 mg/kg/day for 7 days. Norepinephrine (Sigma) was infused into rats at a rate of 2.8 mg/kg/day. In some experiments, rats were given daily subcutaneous injections of the iron chelator, deferoxamine (DFO, a kind gift from Novartis) at a dose of 200 mg/kg/day. Iron overload was induced by an intraperitoneal injection of an iron–dextran complex (a kind gift from Teikoku Hormone Mfg) at a dose of 240 mg of elemental iron/kg on days 0, 2, 4, and 6 of angiotensin II infusion.

In some experiments, a new free radical scavenger, T-0970 [12] (a kind gift from Tanabe Seiyaku), was given orally at a dose of 10 mg/kg/day. Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography (Ueda Seisakusyo). Concentrations of plasma and urine creatinine were measured by the Jaffe reaction, and urinary protein was measured by the pyrogallol red-molybdate protein dye-binding method (SRL).

2.2. Northern blot analysis

Total RNA was obtained using Isogen (Wako), and mRNA was subsequently isolated using oligotex-dT30 (Roche Diagnostics). Mouse *klotho* cDNA was labeled with [α-^{32}P]dCTP (Amersham Life Sciences) 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 a band corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

2.3. Western blot analysis

Polyclonal antibodies against mouse Klotho (a kind gift from Kyowa Hakko) and monoclonal antibody against β-actin (Sigma) were used at dilutions of 1/4000 [9] and 1/2000, respectively. The enhanced chemiluminescence (ECL) Western blotting system (Amersham Life Sciences) was used for detection. Band intensity was calculated and is expressed as a percentage of the control value.

2.4. Measurement of plasma levels of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$)

Plasma free 8-epi-$PGF_{2\alpha}$ was measured by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) as described elsewhere [13]. Briefly, a four-sector type MStation 700 tandem mass spectrometer (JEOL) equipped with an ESI source was used in the negative ion-selected ion-monitoring mode. Quasi-molecular ions (deprotonated ions), *m/z* 353.24 and *m/z* 357.26 for 8-epi-$PGF_{2\alpha}$ and the

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internal standard, respectively, were each monitored for 500 ms each in the selected ion-monitoring mode at a mass spectral resolution of 1500.

2.5. Statistical analysis

Data are expressed as the mean ± S.E.M. We used analysis of variance (ANOVA) followed by a multiple comparison test to compare the initial data, before expressing the results as a percentage of the control value using the statistical analysis software StatView. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of iron overload on renal klotho expression

Iron overload did not significantly increase the blood pressure of rats (131 ± 3 mmHg, $n = 10$) as compared to untreated control rats (131 ± 3 mmHg, $n = 6$). In the kidney of rat subjected to iron overload, expression of *klotho* mRNA was downregulated (Fig. 1) to a similar extent as in rats given a hypertensive dose of angiotensin II (192 ± 4 mmHg, $n = 10$). Concomitant administration of iron–dextran and angiotensin II did not have additive effects on *klotho* expression. When rats were subjected to iron overload and norepinephrine at a dose that exerted hypertensive effects comparative to those of angiotensin II, downregulation of *klotho* was also observed. However, downregulation of *klotho* was not observed in rats given norepinephrine alone. Western blot analysis showed that iron overload also decreased the renal expression of Klotho protein (Fig. 1).

3.2. Effects of iron chelation and a free radical scavenger on angiotensin II-induced downregulation of klotho gene

Treatment of angiotensin II-infused rats with deferoxamine, which did not significantly affect blood pressure (196 ± 7 mmHg, $n = 9$), inhibited angiotensin II-induced downregulation of both *klotho* mRNA and Klotho protein (Fig. 2A, B). Treatment of the angiotensin II-infused rats with a free radical scavenger, T-0970, which again did not significantly affect blood pressure (200 ± 9 mmHg, $n = 11$), also inhibited, albeit partially, angiotensin II-induced downregulation of the *klotho* gene (Fig. 2A, C). Histological analysis showed that treating angiotensin II-infused rats with deferoxamine [10], but not with T-0970 (unpublished data), inhibited iron deposition in the renal tubular epithelial cells.

3.3. In vivo oxidative stress in rats treated with various agents

We investigated the extent of in vivo oxidative stress by measuring plasma levels of 8-epi-$PGF_{2\alpha}$. Angiotensin II administration increased plasma levels of 8-epi-$PGF_{2\alpha}$ as we have reported previously [14]. Iron overload also increased plasma levels of 8-epi-$PGF_{2\alpha}$ to levels similar to those induced by angiotensin II (Fig. 3). The angiotensin II-induced increase in plasma levels of 8-epi-$PGF_{2\alpha}$ was blocked partially by deferoxamine, and completely by T-0970, which verified the use of T-0970 as a free radical scavenger in our system.

3.4. Effect of radical scavenger on the renal function and extent of proteinuria

We have previously demonstrated that the induction of *klotho* expression by adenovirus-mediated gene transfer ameliorates angiotensin II-induced increase in proteinuria [9]. We have also showed that the treatment of angiotensin II-infused rats with deferoxamine suppresses the proteinuric effects of angiotensin II [10]. These results, together with the findings of the present study, suggested that the proteinuric effects of

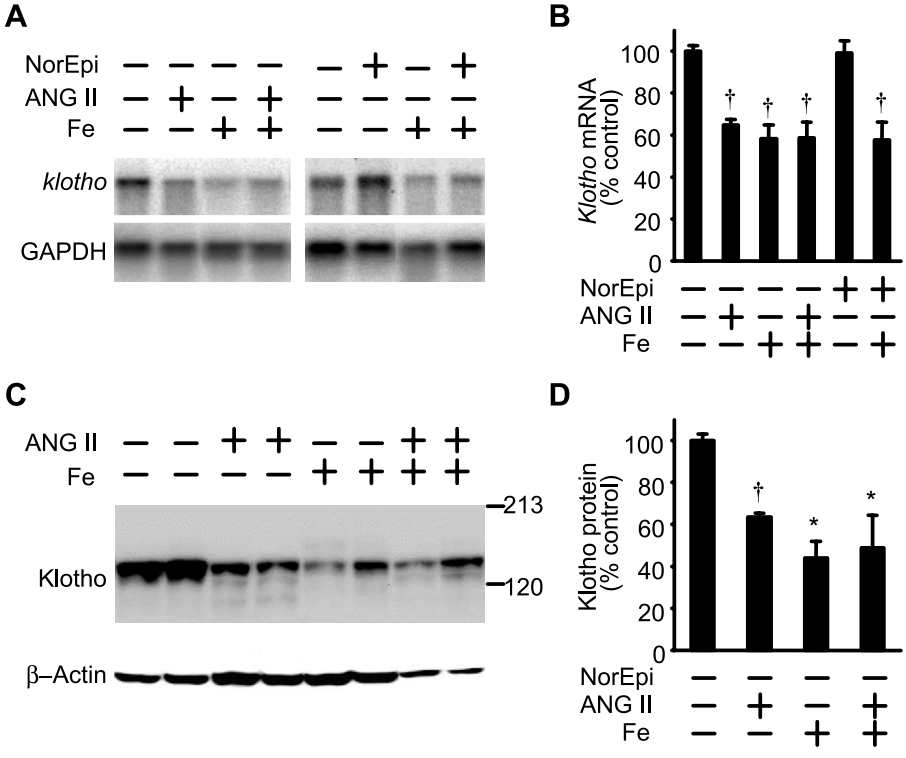

Fig. 1. Expression of *klotho* in the kidney after rats were treated with angiotensin II, norepinephrine, and iron–dextran. A: Representative Northern blot. B: Summary of the Northern blot data from four to six animals in each group. C: Representative Western blot. D: Summary of the Western blot data from four to six animals in each group. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ versus untreated rats.

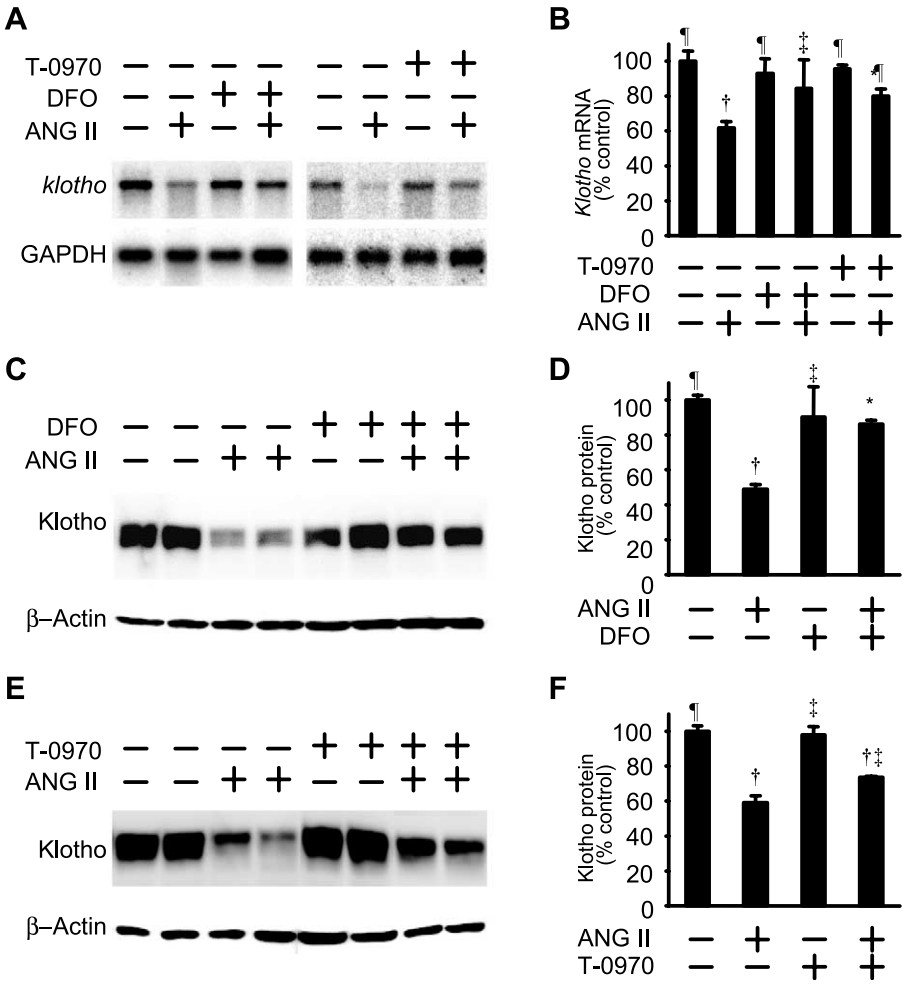

Fig. 2. Effects of an iron chelator and a free radial scavenger on angiotensin II-induced *klotho* downregulation. A: Representative Northern blot. B: Summary of the Northern blot data from four to six animals in each group. C: Effect of deferoxamine on the angiotensin II-induced decrease in Klotho protein. Representative Western blot. D: Summary of the Western blot data from four to six animals in each group. E: Effect of the free radical scavenger T-0970 on the angiotensin II-induced decrease in Klotho protein. Representative Western blot. F: Summary of the Western blot data from four to six animals in each group. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ versus untreated rats, and $^{\ddagger}P < 0.05$ and $^{\P}P < 0.01$ versus angiotensin II-treated rats.

angiotensin II might be augmented by the downregulation of *klotho* expression induced by this octapeptide. Thus, we measured the extent of proteinuria and creatinine clearance in the rats treated with the radical scavenger. As expected, T-0970 suppressed the angiotensin II-induced increase in proteinuria, but did not affect the angiotensin II-induced decrease in creatinine clearance (Fig. 4).

4. Discussion

In the present study, we have shown that iron overload decreased renal expression of the *klotho* gene at both the mRNA and the protein level, and that iron chelation suppressed the angiotensin II-induced downregulation of *klotho*. These findings suggest the mechanisms of angiotensin II-induced downregulation of *klotho* may include abnormal iron metabolism in the kidney elicited by angiotensin II administration. Furthermore, as treating the angiotensin II-infused rats with a free radical scavenger suppressed the angiotensin II-induced downregulation of *klotho*, an increased production of reactive oxygen species may also play some role in the altered *klotho* expression caused by angiotensin II administration. Iron is known to enhance organ damage induced by oxidants by catalyzing the Fenton reaction to generate highly toxic hydroxyl radicals, thus it is possible that the angiotensin II-induced deposition of renal iron may decrease *klotho* by inducing oxidative stress.

Although the phenotypic effects and cardiovascular functions of the *klotho* gene have been extensively studied in animal models, recent studies suggest that this gene may also have a considerable physiological significance in humans because of the possible association between a human variant of *klotho* and both reduced human longevity [4] and susceptibility to atherosclerotic diseases [5]. As *klotho* expression has been shown to be regulated in humans [6], it is important to clarify the mechanism responsible for this regulation.

Expression of *klotho* is downregulated in animal models of hemodynamic and metabolic disorders, such as the spontaneously hypertensive rat, the deoxycorticosterone acetate-salt hypertensive rat, and the Otsuka Long–Evans Tokushima fatty rat [7]. Drug interventions that ameliorate hemodynamic and metabolic abnormalities augment the renal expression of

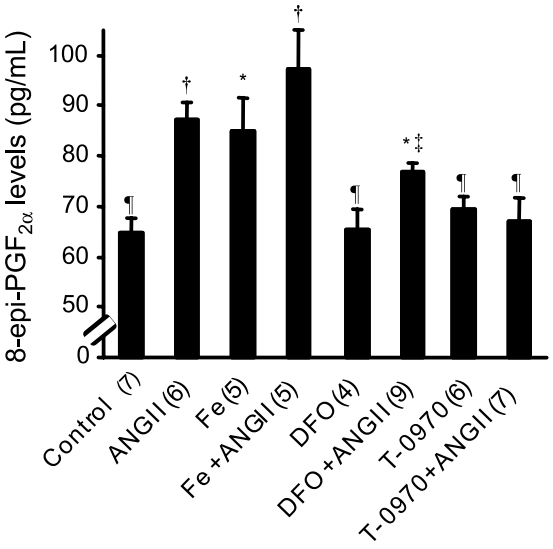

Fig. 3. Plasma levels of 8-epi-$PGF_{2\alpha}$ in the rats treated with angiotensin II, iron–dextran, deferoxamine, and free radical scavenger. Number of each group was written in parentheses. $^{*}P<0.05$ versus untreated rats and $^{\dagger}P<0.05$ versus angiotensin II-treated rats. $^{*}P<0.05$ and $^{\dagger}P<0.01$ versus untreated rats, and $^{\ddagger}P<0.05$ and $^{\P}P<0.01$ versus angiotensin II-treated rats.

klotho in some genetically altered animal models, which suggests that, in addition to the genetic background, factors involved in hemodynamic and metabolic processes may indeed have a role in the regulation of *klotho* expression [15]. Further study of the pathway that regulates *klotho* expression is not easy, however, because to date no established cultured renal cell lines are reported to express this gene.

In the present study, we have shown that a free radical scavenger suppressed the angiotensin II-induced downregulation of *klotho*, which suggests that an increase in systemic oxidative stress may decrease renal *klotho* expression. However, norepinephrine infusion, which also increases plasma levels of 8-epi-$PGF_{2\alpha}$ [14], did not decrease renal expression of *klotho*. Thus, an increase in systemic oxidative stress may not account, or may not be sufficient, for the angiotensin II-induced decrease in renal expression of *klotho*.

In the last part of the present study, we examined the effects of a free radical scavenger on the renal function and on renal excretion of protein in the angiotensin II-infused rats and found that the radical scavenger inhibited the proteinuric effects of angiotensin II. This observation is consistent with the notion that angiotensin II-induced renal damage will be ameliorated by increased expression of *klotho*. Whether previously observed anti-proteinuric effects of antioxidants [16,17] are mediated, or at least accompanied, by the augmentation of renal *klotho* expression should be investigated in future studies.

In conclusion, iron overload decreased renal expression of *klotho* at both the mRNA and the protein level, and iron chelation suppressed the angiotensin II-induced downregulation of this gene, suggesting that alteration in iron metabolism may have a role in the angiotensin II-induced downregulation of renal *klotho* expression. Furthermore, a free radical scavenger also suppressed the angiotensin II-induced downregulation of *klotho*, supporting the involvement of an increased production of reactive oxygen species in this process.

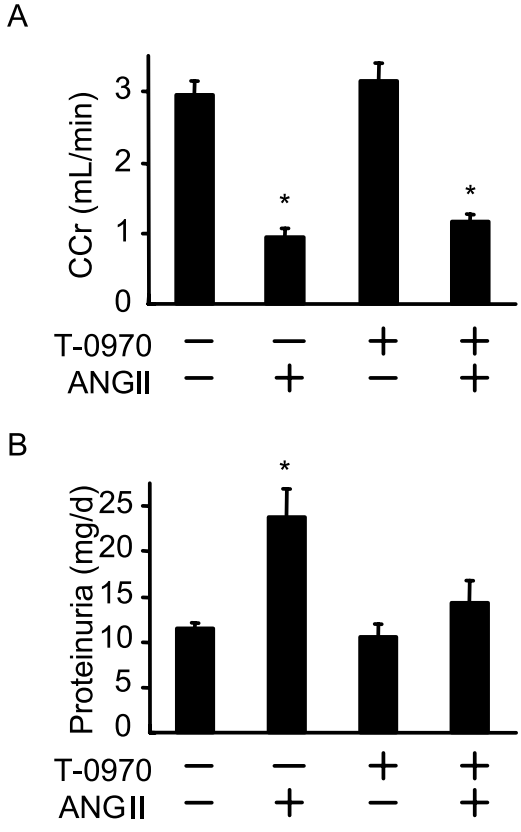

Fig. 4. Renal function and proteinuria in rats treated with administration of angiotensin II and a free radical scavenger. A: Creatinine clearance. B: Proteinuria. Data from five to eight plasma or urine samples are summarized in the table. CCr indicates creatinine clearance. $^{*}P<0.01$ vs. untreated control.

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