Klotho Gene Deficiency Causes Salt-Sensitive Hypertension via Monocyte Chemotactic Protein-1/CC Chemokine Receptor 2–Mediated Inflammation

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ABSTRACT

Klotho (KL) is a newly discovered aging suppressor gene. In mice, the KL gene extends the lifespan when overexpressed and shortens the lifespan when disrupted. This study investigated if KL deficiency affects BP and salt sensitivity using KL mutant heterozygous (+/−) mice and wild-type (WT) mice (9 weeks of age, 16 mice per group). Notably, systolic BP in KL(+/−) mice began to increase at the age of 15 weeks, reached a peak level at the age of 17 weeks, and remained elevated thereafter, whereas systolic BP remained consistent in WT mice. High salt (HS) intake further increased BP in KL(+/−) mice but did not affect BP in WT mice. Blockade of CC chemokine receptor 2 (CCR2), involved in monocyte chemotaxis, by a specific CCR2 antagonist (INCB3284) abolished the HS-induced increase in BP in KL(+/−) mice. Furthermore, HS loading substantially increased the expression of monocyte chemotactic protein-1 and the infiltration of macrophages and T cells in kidneys in KL(+/−) mice, and treatment with INCB3284 abolished these effects. Treatment of KL(+/−) mice with INCB3284 also attenuated the increased renal expressions of serum glucocorticoid-regulated kinase 1, thiazide-sensitive NaCl cotransporter, and ATP synthase β along with the renal structural damage and functional impairment induced by HS loading. In conclusion, KL deficiency caused salt-sensitive hypertension and renal damage by CCR2-mediated inflammation.

In 1997, a new aging suppressor gene was identified and named after the purported Greek Moira Klotho,1 who spins the thread of life and controls the ultimate destiny of humans. Insertional mutation of mouse klotho gene resulted in extensive premature aging phenotypes and shortened lifespan.1 However, overexpression of mouse klotho gene extended the lifespan by 20%–30% and rescued aging disorders.2 Therefore, klotho is an antiaging gene.

Aging is characterized by age-related deterioration in health. Hypertension is a common aging-related disorder. The prevalence of hypertension increases with age.3 On the basis of an epidemiologic study,4 the prevalence of hypertension is more than doubled in the elderly than the young population. More than two thirds of individuals over age 65 years suffer from hypertension according to the Seventh Report of the Joint National Committee.4

Salt intake is one of the main environmental factors contributing to the development of hypertension. An increase in salt sensitivity has been noted with advancing age in numerous studies.5–9 Salt sensitivity is more common in the old than the young population. The relationship of age and salt sensitivity seems to be stronger in hypertensive than
normotensive individuals. It was reported that salt sensitivity was present in approximately 50% of hypertensive individuals <40 years old but increased to over 80% in those >60 years old. Xiao et al. reported that the klotho level in serum declines with age in humans after 40 years of age. Although the klotho level decreases and the salt sensitivity increases with age, it is not clear if klotho deficiency enhances salt sensitivity.

The objective of this study is to investigate if one-half klotho deficiency affects salt sensitivity and investigate the underlying mechanism in klotho mutant heterozygous (+/− [KL(+/−)]) mice.

Monocyte chemotactic protein-1 (MCP-1), belonging to the C-C chemokine family, can be produced by many cell types in response to inflammatory stimuli, such as TNF-α, IL-1, and IFN-γ. MCP-1 plays a key role in regulating migration and infiltration of monocytes/macrophages and memory T cells by binding to its specific cell surface receptor (CC chemokine receptor 2 [CCR2]). CCR2 is highly expressed in monocytes. Other studies showed that CCR2 was also expressed on activated and memory T cells, including both T helper 1 (Th1) and T helper 2 (Th2) cells. Therefore, we assessed if klotho deficiency causes renal inflammation and investigated if MCP-1/CCR2 plays a role in inflammation and salt sensitivity in KL(+/−) mice.

The progressive renal recession is also a common phenomenon in the aging process. The association of the age-related nephropathy and renal function decline has been confirmed, even after excluding other confounding factors. Nevertheless, how aging deteriorates kidney function remains unknown. The klotho protein is primarily expressed in kidneys, predominately in distal convoluted tubules. Recent studies showed renal and systemic klotho deficiency in both AKI and CKD. Klotho deficiency may serve as an early biomarker as well as a pathogenic factor for the progression of renal disease and additional complications.

At age 70 years, the serum level of klotho is about one half of what it was at age 40 years. Because the klotho level decreases with age, whereas renal function declines with age, we investigated if one-half klotho deficiency causes renal damage, with a focus on the underlying mechanism. Specifically, we assessed if CCR2-mediated monocyte chemotaxis is involved in renal damage in KL(+/−) mice.

RESULTS

One-Half Klotho Deficiency (+/−) Caused Spontaneous Hypertension and Increased Salt Sensitivity

Western blot analysis indicated that klotho protein expression in kidneys of KL(+/−) mice is about one half of that of wild-type (WT) mice (Supplemental Figure 1). Systolic BP began to increase spontaneously around 15 weeks of age (Figure 1A). Systolic BP was significantly and persistently elevated in KL (+/−) mice from the age of 16 weeks and remained elevated thereafter. Therefore, one-half klotho deficiency causes spontaneous hypertension.

Systolic BP was also remarkably increased in KL(+/−) mice after receiving 2% saline intake (Figure 1B). Although BP was slightly elevated in KL(+/−) mice in response to 1% saline, it did not reach a significant level. High-salt (HS) intake (1% or 2%) had no effects on BP in WT mice. The results revealed, for the first time, that one-half klotho deficiency increased salt sensitivity and caused salt-sensitive hypertension.

Blockade of CCR2 Abolished HS-Induced Hypertension in KL(+/−) Mice

Daily intraperitoneal injection of INCB3284 (15 mg/kg), a CCR2-selective antagonist, abolished HS-induced elevation of BP in KL(+/−) mice but did not decrease it to the level of WT mice (Figure 1C). INCB3284 did not affect BP in KL(+/−) mice receiving regular water. INCB3284 had no effects on systolic BP of WT mice in the absence or presence of 2% saline. Blockade of CCR2 by another CCR2-selective antagonist, RS102895, also eliminated HS-induced increase in BP but did decrease it to the level of WT mice (Supplemental Figure 2). These results showed that the CCR2-mediated pathway was involved in salt sensitivity and salt-induced hypertension in KL(+/−) mice but not in spontaneous elevation of BP in KL(+/−) mice.

HS Loading Further Upregulated MCP-1 in Kidneys of KL(+/−) Mice

The protein expressions of MCP-1 and TNF-α in kidneys were upregulated in KL(+/−) mice (Figure 2, A–D). Remarkably, they were further increased by HS loading, suggesting that the HS loading exacerbated klotho deficiency–induced inflammatory activation in kidneys. In contrast, the HS loading did not alter the expression of MCP-1 and TNF-α significantly in WT mice (Figure 2, A–D). Blockade of CCR2 by INCB3284 did not affect the expression of MCP-1 or TNF-α in either KL(+/−) or WT mice, regardless of HS or regular water intake. One-half klotho deficiency or INCB3284 did not affect CCR2 protein expression in kidneys (Figure 2, E and F).

HS Further Increased Infiltration of Macrophages and T Cells in Kidneys of KL(+/−) Mice, Which Can Be Abolished by INCB3284

The immunostaining showed infiltration of macrophages (CD68) and T cells (CD4 and CD8) in kidneys. The numbers of CD68+, CD4+, and CD8+ cells were increased in kidneys of KL(+/−) mice versus WT mice (Figures 3B and 4, B and D), indicating that one-half klotho deficiency increased infiltration of macrophages and T cells in kidneys. INCB3284 abolished macrophage (CD68+) infiltration in kidneys of KL(+/−) mice (Figure 3B), suggesting effective blockade of CCR2. Therefore, klotho deficiency–induced macrophage infiltration was mediated by CCR2. Although INCB3284 significantly attenuated infiltration of T lymphocytes (CD4+ and CD8+) in KL(+/−) mice, it did not decrease it to the WT level (Figure 4). The results suggest that klotho deficiency–induced T-cell infiltration was largely, but not completely, mediated by the MCP-1/CCR2 pathway.
HS exacerbated infiltration of inflammatory cells in kidneys of KL(+/−) mice (Figures 3 and 4). INCB3284 abolished the HS-induced increase in T cells and macrophages in kidneys of KL(+/−) mice (Figure 3B and 4, B and D). This result suggests that the HS-induced infiltration of inflammatory cells was fully CCR2-dependent.

**Blockade of CCR2 by INCB3284 Abolished HS-Induced Upregulation of the Serum Glucocorticoid-Regulated Kinase 1–Thiazide-Sensitive NaCl Cotransporter Signaling and ATP Synthase β in Kidneys of KL(+/−) Mice**

One-half klotho deficiency or INCB3284 did not affect mineralocorticoid receptor (MR) protein expression in kidneys (Figure 5, A and B). The protein expressions of serum glucocorticoid-regulated kinase 1 (Sgk1), thiazide-sensitive NaCl cotransporter (NCC), and ATP synthase β were upregulated in kidneys of KL(+/−) mice versus WT mice (Figure 5, C–H). Interestingly, protein expressions of these signaling molecules, remarkably, were further increased by HS loading in KL(+/−) mice but not WT mice.

Blockade of CCR2 by INCB3284 significantly attenuated the upregulation of Sgk1, NCC, and ATP synthase β in KL(+/−) mice, although the expression of Sgk1 and NCC in KL(+/−)-INCB mice remained higher than that of WT mice (Figure 5, C–H). Notably, INCB3284 abolished the HS-induced increases in protein expression of Sgk1, NCC, and ATP synthase β in KL(+/−) mice (Figure 5, C–H).

**Blockade of CCR2 by INCB3284 Abolished HS-Induced Renal Structural Damage in KL(+/−) Mice**

Tubular dilation and atrophy, as well as tubular collapse, were found in some medullary regions of KL(+/−) mice, which...
were exacerbated by HS loading in KL(+/-) mice (Figure 6A). In contrast, these pathologic changes were not seen in age-matched WT and WT-HS mice. The results showed that one-half klotho deficiency caused renal tubular damage, which was further deteriorated by HS loading. INCB3284 attenuated tubular structural damage and abolished HS-induced exacerbation in KL(+/-) mice (Figure 6A).

The Masson trichrome staining showed more collagen deposition in renal tubular interstitium in KL(+/-) mice versus WT mice (Figure 6, B and C), indicating that one-half klotho deficiency caused tubulointerstitial fibrosis. HS loading exacerbated fibrotic damage in KL(+/-) mice. INCB3284 attenuated fibrotic damage in KL(+/-) mice. INCB3284 completely abolished HS-induced exacerbation of fibrotic formation.

Therefore, CCR2-mediated inflammation was involved in klotho deficiency–induced and HS-exacerbated renal tubular damage and tubulointerstitial fibrosis.

Figure 2. HS loading further upregulated MCP-1 in kidneys of KL(+/-) mice. (A) Representative Western blots and (B) quantitative analysis of MCP-1 in kidneys. (C) Representative Western blots and (D) quantitative analysis of TNF-α protein expression in kidneys. (E) Representative Western blots and (F) quantitative analysis of CCR2 protein expression in kidneys. Western blots analyses were performed when animals were euthanized at 10 days after treatment. The relative protein expression was first normalized to β-actin and then calculated as fold changes of the controls (WT). Data=means±SEMs (n=4 mice/group). *P<0.05 versus KL(+/-); **P<0.01, ***P<0.001 versus WT; #P<0.05 versus KL(+/-)-HS.

Blockade of CCR2 by INCB3284 Abolished HS-Induced Renal Functional Impairment in KL(+/-) Mice

Hematoxylin and eosin (HE) staining in kidneys showed tubular cast (protein) formation, a sign of severe kidney damage, in some local medullary regions of KL(+/-) mice but not in WT mice (Figure 7, A and B). Tubular cast formation, remarkably, was further increased by HS loading in KL(+/-) mice. INCB3284 attenuated tubular cast formation and eliminated HS-induced exacerbation of cast formation in KL(+/-).

Renal glomerular impairment in KL(+/-) mice was evidenced by significant increases in urine albumin levels, which were worsened by HS (Figure 7C). INCB3284 significantly decreased urine albumin levels in KL(+/-) and KL(+/-)-HS mice, suggesting improved glomerular function. Plasma urea concentrations were increased in KL(+/-) mice, which were abolished by INCB3284 (Figure 7D). HS tended to further increase plasma urea concentrations in KL(+/-) mice, which were attenuated by INCB3284 treatments, although the
data did not reach significance levels (Figure 7D). Blockade of CCR2 by another CCR2-selective antagonist, RS102895, abolished the HS-induced increase in plasma urea in KL(+/−) mice (Supplemental Figure 3B). Serum creatinine was increased by HS, which could be significantly attenuated by RS102895 (Supplemental Figure 3A). These results indicated that MCP-1/CCR2-mediated inflammation may be involved in impaired renal function in KL(+/−) mice.

DISCUSSION

This study revealed that systolic BP was elevated spontaneously and persistently in KL(+/−) mice after the age of 16 weeks (Figure 1A), suggesting that the klotho gene plays an important role in the maintenance of normal BP. Klotho is an aging suppressor gene.1,2 In humans, the klotho level decreases with age,3 whereas the prevalence of hypertension increases with age.4 The prevalence of hypertension in the elderly population is two times as much as that of the young population.3 A clinical study showed an association between G-395A polymorphism in the promoter of the Klotho gene and essential hypertension in the Chinese Han population, especially in subjects over 60 years old.27 However, the −395A variant of the G-395A single-nucleotide polymorphism might protect against the development of essential hypertension by upregulating Klotho gene expression, because the A allele has higher promoter activity in vitro.27 Therefore, klotho deficiency may be a candidate etiologic factor for essential hypertension. Our recent studies showed that adenovirus-mediated klotho gene delivery reduced elevated BP in Otsuka Long–Evans Tokushima Fatty rats. Therefore, supplement of klotho might be a potential therapeutic strategy for hypertension.

Interestingly, BP of KL(+/−) mice was further elevated by HS loading, which however, did not affect BP in WT mice (Figure 1B). Thus, this study showed, for the first time, that klotho deficiency increased salt sensitivity and elicited salt-sensitive hypertension. In humans, the klotho level is decreased,11 whereas the salt sensitivity is increased in the elderly hypertensive population.5,6,8 Thus, a decrease in klotho may be a candidate etiologic factor for the enhanced salt sensitivity and salt–sensitive hypertension in the elderly population. Notably, blockade of CCR2, a receptor of MCP-1, by INCB3284 or RS102895 abolished the HS-induced increase in BP in KL(+/−) mice (Figure 1C, Supplemental Figure 2), suggesting that the CCR2-mediated inflammation may play an important role in klotho deficiency–induced salt–sensitive hypertension.

The expression of MCP-1 and infiltration of macrophages and T cells were upregulated in kidneys of KL(+/−) mice (Figures 2–4). Notably, these inflammatory responses were further markedly increased by HS loading. Although CCR2 expression was not different between KL(+/−) and WT mice and not affected by HS loading (Figure 2E), MCP-1 expression was further upregulated in kidneys of KL(+/−) mice by HS loading (Figure 2A). MCP-1 activates the chemotaxis activity by binding to CCR2.16,17 Blockade of CCR2 by INCB3284, a potent selective antagonist of CCR2, abolished HS-induced infiltration of both macrophages and T cells in kidneys of KL(+/−) mice (Figures 3 and 4), suggesting that the upregulation of MCP-1 may mediate klotho deficiency–induced renal inflammation by CCR2. Nevertheless, how klotho deficiency causes the upregulation of MCP-1 is incompletely understood. The in vitro studies showed that exogenous addition or overexpression of klotho suppressed TNF-α–stimulated activation of NF-κB and subsequent production of cytokines, such as...
MCP-1, by inhibition of RelASer phosphorylation. Additional study is required to assess whether klotho deficiency directly increases MCP-1 expression. However, plasma aldosterone levels were elevated in KL(+/−) mice (Supplemental Figure 4). We showed recently that upregulation of plasma aldosterone levels may be involved in klotho deficiency–related renal inflammation, which could be abolished by blockade of aldosterone receptors by eplerenone. Eplerenone also attenuated kidney damage and improved kidney function. Klotho deficiency increased expression of adrenal CYP11B2, a key enzyme for aldosterone synthesis. Therefore, upregulation of aldosterone may be a factor contributing to klotho deficiency–induced increases in MCP-1/immune cell infiltration.

The infiltration of inflammatory cells in kidneys was found in salt-sensitive hypertension. Franco et al. also reported an inverse correlation between the number of renal tubulointerstitial inflammatory cells (lymphocytes and macrophages) and the urinary sodium excretion in salt-sensitive hypertensive rats. Mattson and colleagues found that T cells may mediate Dahl salt-sensitive hypertension.

It is noted that, although blockade of CCR2 by RS102895 abolished HS-induced hypertension (Supplemental Figure 2) and improved kidney function (Supplemental Figure 3), it did not alter vascular relaxing responses to acetylcholine or sodium nitroprusside in KL(+/−) mice on HS diet (Supplemental Figure 5). This result suggests that the antihypertensive effect of blockade of CCR2 may not be caused by vasorelaxant effects of this compound. Klotho is predominately expressed in kidneys. There is no convincing data showing that klotho is expressed in vascular cells.

The control of sodium excretion is achieved by regulating sodium transport in renal tubules. NCC is the predominant apical sodium transporter located in the distal convoluted tubule, which has the highest sodium-potassium-ATPase activity among all renal tubule segments. The abundance and activity of NCC are modulated by the With-no-lysine (WNK) family

Figure 4. HS further increased infiltration of T cells in kidneys of KL(+/−) mice, which can be abolished by INCB3284. (A) Representative photomicrographs of CD4 immunostaining in kidney sections (arrows indicate CD4+ T cells). (B) Semiquantitative analysis of infiltration of CD4+ T cells in kidneys. (C) Representative photomicrographs of CD8 immunostaining in kidney sections (arrows indicate CD8+ T cells). (D) Semiquantitative analysis of infiltration of CD8+ T cells in kidneys. Immunostaining was performed when animals were euthanized at 10 days after treatment with INCB3284. Scale bars, 50 μm. Data=means±SEMs. ***P<0.001 versus KL(+/−); *P<0.05, **P<0.01, ***P<0.001 versus WT; ###P<0.001 versus KL(+/−)-HS.
of serine/threonine kinases. WNK4 can reduce NCC expression on the cell surface by disrupting NCC trafficking to the apical membrane. Sgk1 can reverse the inhibitory effect of WNK4 on NCC by binding to and phosphorylating WNK4, which leads to an increasing net effect of NCC. This study showed that the expressions of Sgk1, NCC, and ATP synthase were upregulated in KL(+/-) mice and further enhanced by HS loading, which can be effectively attenuated by INCB3284 (Figure 5). Nevertheless, this study cannot assess the functional significance of increased expression of NCC and Sgk1. It is expected that the upregulation of NCC/Sgk1 may lead to increased Na reabsorption and positive salt balance through their known function in Na reabsorption, which could be attenuated by blockade of CCR2. This hypothesis, however, needs to be validated by measuring urinary Na excretion.

The Sgk1-NCC pathway has been established as an important downstream target of aldosterone/MR. In theory, HS loading should suppress the circulating aldosterone level and MR downstream signaling activation for keeping sodium balance. Indeed, HS loading slightly decreased plasma aldosterone levels (Supplemental Figure 4). However, the MR downstream Sgk1-NCC signaling was upregulated by HS loading in KL(+/-) mice, which can be blocked by INCB3284 (Figure 5). This result suggests that the CCR2-mediated inflammatory process interfered with the negative feedback regulation of the MR-dependent cascades in response to HS loading, although the underlying mechanism remains to be elucidated. Several studies indicated that some cytokines, such as TNF-α and IL-6, were capable of activating Rac1, a member of the Rho family of GTPases. Rac1 has been proven to play a pivotal role in activation of the Sgk1 signaling by salt...
excess independent of aldosterone by promoting MR nuclear translocation.45

Although the infiltration of inflammatory cells and the upregulation of the Sgk1-NCC signaling in kidneys of KL (+/−) mice were attenuated or abolished by INCB3284 (Figures 3–5), the elevated BP level in KL(+/−) mice was not decreased by INCB3284. The results suggest that the spontaneous elevation of BP in KL(+/−) mice may be mediated by a mechanism other than inflammation in kidneys. The spontaneous elevation of BP was investigated in our recent study, which revealed that plasma level of aldosterone was increased in KL(+/−) mice.32 The elevated BP of KL(+/−) mice was decreased to the WT level after treatment with eplerenone,32 a specific MR antagonist, suggesting that the spontaneous elevation of BP in KL(+/−) mice was caused by hyperaldosteronism. Eplerenone attenuated klotho deficiency–induced inflammation in kidneys. This study also showed that basal level of plasma aldosterone was elevated in KL(+/−) mice (Supplemental Figure 4). Aldosterone/MR promotes hypertension by not only increasing inflammation and sodium reabsorption through the Sgk1-ENaC/NCC pathway42 but also, its actions on angiotensin II,46 endothelial function,47 vascular compliance,48 and central nervous system.49

Arginine vasopressin (AVP) has been reported to play an important role in the development of deoxycorticosterone acetate salt hypertension.50 AVP is also involved in salt-sensitive hypertension in Dahl salt-sensitive rats.51 It was recently reported that γ-aminobutyric acid–ergic excitation of vasopressin neurons in the supraoptic nucleus is a critical mechanism underlying sodium–dependent hypertension.52 Therefore, additional study is warranted to assess if klotho deficiency promotes AVP synthesis and release in response to HS, which could contribute to the development of klotho deficiency–induced salt-dependent hypertension.

Another interesting finding was that one-half klotho deficiency caused renal structural damage and renal functional

Figure 6. Blockade of CCR2 by INCB3284 abolished HS-induced renal structural damage in KL(+/−) mice. (A) Representative photomicrographs of HE-stained kidney sections. Tubular damages, including dilation and atrophy of tubules, as well as loss of tubular structure (indicated by arrows), were observed in some local regions of medulla in KL(+/−) mice. These structural damages were exacerbated by HS in KL(+/−) mice. INCB3284 attenuated these local medullary tubular injuries. (B) Representative photomicrographs of Masson trichrome–stained kidney sections (blue staining indicates collagen deposition). (C) Semiquantitative analysis of collagen staining in tubular interstitium. Both stainings were performed when animals were euthanized at 10 days after treatment with INCB3284. Scale bars, 50 μm. *P<0.05, **P<0.01, ***P<0.001 versus KL(+/−); †P<0.05, ‡P<0.01 versus WT; §§P<0.001 versus KL(+/−)-HS.
imPAIRMENTS, WHICH WERE EXACERBATED BY SALt EXCESS (FIGURES 6 AND 7). Klotho deFICIENCY–INDUCED RENAL DAMAGE WAS LARGELy ATTRIBUTED TO THE CCR2-MEDIATED INFLAMMATORY CELL INFILTRATION, BECAUSE IT CAN BE ATTENUATED BY INCB3284. NOTABLY, INCB3284 SIGNIFICANTLY IMPROVED RENAL DAMAGE IN KL(+/-) MICE, ALTHOUGH IT DID NOT DECREASE THE ELEVATED BP TO THE CONTROL LEVEL, SUGGESTING THAT KLOTHO DEFICIENCY–INDUCED KIDNEY DAMAGE MAY BE INDEPENDENT OF HYPERTENSION. THIS NOTION IS SUPPORTED BY OUR RECENT FINDING THAT KLOTHO GENE DELIVERY ABOLISHED KIDNEY DAMAGES IN SPONTANEOUS HYPERTENSIVE RATS, WHEREAS BP REMAINS ELEVATED. Klotho suppressed Nox2-mediated superoxide generation, contributing to its renoprotective effect.28

In summary, this study showed, for the first time, that klotho deficiency caused salt-sensitive hypertension by activating the CCR2-mediated inflammatory process. The inflammatory cytokine activation and subsequent inflammatory cell infiltration upregulated the Sggk1-NCC signaling in kidneys in response to HS loading and resulted in renal structural injury and renal functional impairment. Therefore, klotho supplement and inflammation suppression may be potential therapeutic strategies for the treatment of salt-sensitive hypertension and kidney damage in elderly patients.

CONCISE METHODS

Animal Study Protocols
KL(+/-) mice in 129Sv background were provided by Makoto Kuro-o.1 The WT littermate 129Sv mice were used as controls. All mice were housed in cages at room temperature (25°C±1°C) and were provided with Purina laboratory chow (no. 5001) and tap water ad libitum. Details are in Supplemental Material.

In total, 16 KL(+/-) mice and 16 WT mice (9 weeks; n=16) were used. BP was measured weekly from the age of 9–23 weeks. After BP was stabilized (5 months), each strain of mice was divided into two groups. One group of each strain received 1% saline followed by 2% saline as drinking fluid, whereas the remaining group of each strain received 2% saline followed by 1% saline.
received regular tap water. BP and body weight were measured two times per week. After the BP level was stable again, each group was further divided into two subgroups at the age of 6 months (n=4). Under each diet condition in each strain, one subgroup received INCB3284 (15 mg/kg per day intraperitoneally; Tocris Bioscience, Bristol, UK), whereas the other subgroup received an equal dose of vehicle (DMSO) and served as a control. BP and body weight were measured two times per week. Urine was collected three times for assessing renal function (urea, creatinine, and albumin) during the treatment. After 10 days of the treatment, animals were euthanized (halothane). Blood was collected for measuring urea and creatinine concentrations. Animals were then perfused transcardially using heparinized saline. One kidney was collected and embedded in paraffin for histologic and immunohistochemical analysis. The other kidney was saved in −80°C for molecular assays.

**Measurements of BP**
BP was measured by the volume-pressure recording (VPR) tail-cuff method with slight warming (28°C) but not heating of the tail using a CODA 6 BP Monitoring System (Kent Scientific). This method has been validated by using a telemetry system.54,55 Animals were gently handled and well trained for the VPR tail-cuff measurement to minimize the handling stress. No signs of stress were observed during BP measurements. The operator was also strictly trained for the measurement procedure. At least 20 stable cycle data were obtained for measurements. The analysis of the result of each animal at every measurement time. The VPR tail-cuff procedure can reliably monitor BP and is a common method for monitoring BP in our laboratory.28,56 Measurements were repeated in time. All other statistical analyses were performed using one-way ANOVA repeated in time. The unpaired t test was used for comparisons between two groups. Tukey multiple-comparison tests were used to assess the significance of differences between means. Significance was set at a 95% confidence limit.

**Morphologic and Immunohistochemical Investigations**
Paraffin-embedded kidney sections (5 μm) were processed for the following staining. At least three random fields of each section were observed and analyzed (15 sections).

**Immunohistochemical Staining**
Staining was performed as described previously.28 Briefly, the sections were incubated overnight (4°C) with primary antibodies against mouse monoclonal (KPI) CD68 (1:100; Abcam, Inc., Cambridge, MA), CD4 (GK1.5; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and CD8-α (H-160) (1:100; Santa Cruz Biotechnology). Subsequently, the sections were incubated with secondary antibody, including goat anti-mouse, goat anti-rabbit, and chicken anti-rat IgG- horseradish peroxidase (1:1000–1:2000; Santa Cruz Biotechnology) for 1 hour. The sections stained without the primary antibody served as negative controls. The sections were examined and photographed at equal exposure conditions and magnification using a Nikon Eclipse Ti-U microscope coupled with a digital color camera. The numbers of CD68+, CD4+, and CD8+ cells infiltrated in kidneys were directly counted under the microscope at equal magnification (×400) under a Nikon Eclipse Ti-U microscope. Tubular cast formation was defined as the red deposition of proteinaceous material in renal tubules. The semiquantitative analysis of relative tubular cast area fraction in medulla (percentage of cast area over the total area in a given field) was measured using NIS-Elements BR 3.0 software (Nikon, Melville, NY).

**Masson Trichrome Staining**
Trichrome staining was performed in kidney sections for detecting renal fibrosis. The blue staining indicated collagen deposition. The semiquantitative analysis of relative collagen area fraction (percentage of blue-stained collagen area over the total area in one field) was measured using NIS-Elements BR 3.0 software.

**Measurements of Renal Function**
Plasma urea level was detected with a quantichrom urea assay kit (DIUR-500; BioAssay Systems, Hayward, CA) according to the manufacturer’s instruction. Urine creatinine level was detected with a quantichrom creatinine assay kit (DICR-500; BioAssay Systems) according to the manufacturer’s instruction. Urine albumin concentration was measured with a mouse-specific microalbuminuria ELISA kit (Albuwell M; Exocell, Philadelphia, PA) according to the manufacturer’s instruction. Urine albumin excretion was normalized to urine creatinine.

**Western Blotting Analyses**
Western blotting analysis was performed as described previously.28 Briefly, the membranes were blocked in 3% BSA or 5% milk in Tris-buffered saline solution/Tween 20 for 2 hours and then incubated overnight (4°C) with primary antibodies against MCP-1 (1:500; Cell Signaling Technology, Danvers, MA), TNF-α (1 μg/ml; Abcam, Inc.), CCR2 (1 μg/ml; Abcam, Inc.), MR (1:250; Abcam, Inc.), Sgk1 (1:500; Santa Cruz Biotechnology), NCC (1:1000; EMD Millipore, Billerica, MA), ATP synthase b (1:20,000; BD Transduction Laboratories Inc., Mississauga, ON, Canada), and β-actin (1:7500; Abcam, Inc.). Goat anti-mouse or goat anti-rabbit with horseradish peroxidase (1:2000–1:15,000; Santa Cruz Biotechnology) was used as a secondary antibody and incubated for 1 hour at room temperature. Proteins were visualized by enhanced chemiluminescence, exposed to an x-ray film, and developed with an x-ray processor (SRA-101A; Canon). The relative protein expression of each sample was first normalized to β-actin and then calculated as fold change of the controls (WT).

**Statistical Analyses**
BP was analyzed using one-way ANOVA repeated in time. All other data were analyzed by one-way ANOVA. The unpaired t test was used for comparisons between two groups. Tukey multiple-comparison tests were used to assess the significance of differences between means. Significance was set at a 95% confidence limit.

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DISCLOSURES
None.

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