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GRK4-mediated adiponectin receptor-1 phosphorylative desensitization as a novel mechanism of reduced renal sodium excretion in hypertension

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Abstract

Hypertensive patients have impaired sodium excretion. However, the mechanisms are incompletely understood. Despite the established association between obesity/excess adiposity and hypertension, whether and how adiponectin, one of the adipokines, contributes to impaired sodium excretion in hypertension has not been previously investigated. The current study tested the hypothesis that adiponectin promotes natriuresis and diuresis in the normotensive state. However, impaired adiponectin-mediated natriuresis and diuresis are involved in pathogenesis of hypertension. We found that sodium excretion was reduced in adiponectin knockout (Adipo$^{-/-}$) mice; intrarenal arterial infusion of adiponectin-induced natriuresis and diuresis in Wistar–Kyoto (WKY) rats. However, the natriuretic and diuretic effects of adiponectin were impaired in

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Author Contribution

K.C., X.-L.M., and C.Z. contributed to overall conception and design of the manuscript, drafting the article or revising it for important intellectual content, final approval of the version to be published, and agreed to be accountable for all aspects of the work. Y.Z., S.W., H.H., A.Z., C.Z., Y. Huang, S.Z., and H.R. performed experiments and contributed to acquisition of data, analysis and interpretation of data, drafting and revising the article, and agreed to be accountable for all aspects of the work. Y. Han, P.A.J., and Y.W. provided valuable comments and reagents, analyzed the data, edited the manuscript, and agreed to be accountable for all aspects of the work. All authors proofread and approved the manuscript.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

spontaneously hypertensive rats (SHRs), which were ascribed to the hyperphosphorylation of adiponectin receptor and subsequent uncoupling from Gαi. Inhibition of adiponectin receptor phosphorylation by a specific point mutation restored its coupling with Gαi and the adiponectin-mediated inhibition of Na^+-K^+-ATPase activity in renal proximal tubule (RPT) cells from SHRs. Finally, we identified G protein-coupled receptor kinase 4 (GRK4) as a mediator of adiponectin receptor hyperphosphorylation; mice transgenic for a hyperphosphorylating variant of GRK4 replicated the abnormal adiponectin function observed in SHRs, whereas down-regulation of GRK4 by renal ultrasound-directed small interfering RNA (siRNA) restored the adiponectin-mediated sodium excretion and reduced the blood pressure in SHRs. We conclude that the stimulatory effect of adiponectin on sodium excretion is impaired in hypertension, which is ascribed to the increased renal GRK4 expression and activity. Targeting GRK4 restores impaired adiponectin-mediated sodium excretion in hypertension, thus representing a novel strategy against hypertension.

Introduction

Hypertension is a major modifiable risk factor for cardiovascular and renal diseases. Approximately 7.6 million premature deaths each year and 92 million disability-adjusted life years, in the world, are attributed to high blood pressure [1]. However, the mechanisms of the pathogenesis of hypertension are still not clear. The interactions among blood pressure-regulating tissues/organs and adipose tissue have received considerable attention [2]. It is known that excessive adiposity accounts for 65–75% of primary hypertension, while 60–76% of patients, who are overweight or obese, have hypertension [3]. Of those possible mechanisms are adipokines, among which adiponectin is an important one. Adiponectin is an adipocytokine that is exclusively produced by adipose tissue, the plasma concentration of which is decreased in subjects with hypertension [4,5]. Decreased serum adiponectin level is a risk factor for hypertension [5]. Moreover, adiponectin knockout ($Adipo^{-/-}$) mice on a high-salt diet develop hypertension [6], which further suggests the importance of adiponectin in hypertension.

The kidney plays a central role in the pathogenesis of hypertension [3,7–9]. Sodium balance is important in the long-term regulation of blood pressure [3,7–9]. Whether adiponectin is involved in the regulation of blood pressure in hypertension is unknown. We hypothesize that the natriuretic and diuretic effects of adiponectin are impaired in hypertension. Therefore, we studied the effect of adiponectin on sodium excretion in the normotensive Wistar–Kyoto (WKY) rat and spontaneously hypertensive rat (SHR) and investigated the underlying mechanism leading to adiponectin dysfunction, if any, in the kidney.

Materials and methods

Materials and animals

Mouse (Catalog#: 4902) and rat (Catalog#: 4903) adiponectin proteins were from Biovision.

$Adipo^{-/-}$ mice and their wildtype controls (WT) were from the Shanghai Biomodel Organism Science and Technology Development Company (China). Tail-derived DNA was

used for genotyping by PCR using the following primers: Wildtype Forward: GGCTCTCTGGGAGAGGCGAGT; Wildtype Reverse: CCATCACGGCCTGGTGTGCC; Mutant Reverse: TTCGCCATTCAGGCTGCGCA.

In vivo studies

All the *in vivo* studies were performed at the Animal Center of The Third Military Medical University. Four to twelve-week-old WKY rats and SHRs (Vital River Laboratory Animal Technology Co. Ltd, Beijing, China) were fed normal diet prior to the performance of the experiments. The rats were anesthetized with pentobarbital (50 mg/kg body weight, intraperitoneally), and then placed on a heating pad to maintain rectal temperature between 36 and 37°C. Anesthesia was maintained by the intravenous infusion of pentobarbital at 0.8 mg/100 g body weight per hour. Catheters were inserted into the external jugular vein and carotid artery, using color closed-circuit surveillance cameras (Panasonic, Osaka, Japan). Systemic arterial pressure was monitored electronically (Bene View T5, Mindray Corporation, Shenzhen, China). A laparotomy was performed, and both the right and left ureters were catheterized (PE-10). The right renal artery was exposed, and the right suprarenal artery, which originates from the right renal artery, was catheterized (PE-10 heat-stretched to 180 μm). Then, the vehicle (saline) or recombinant rat adiponectin (0.1–10 μg/kg/min, Biovision Life Science Source Inc., San Francisco, CA) was infused at a rate of 40 μl per hour into the right suprarenal artery. Fluid losses during surgery were replaced with 5% albumin at 1% body weight over 30 min, followed by a rate of 0.8 ml/100 g body weight per hour until the end of the experiment, as previously reported [10–12]. After an equilibration period of 120 min, urine was collected from the catheterized ureter every 40 min.

Human G protein-coupled receptor kinase 4 (hGRK4)γ wildtype (WT) and hGRK4γ 142A>V transgenic mice were generated in C57BL/6 background. Adipo$^{-/-}$ (adiponectin deficient) mice were generated in B6.129 background, as previously described [13–15]. The mice were anesthetized with pentobarbital (50 mg/kg body weight, intraperitoneally). To observe the function of adiponectin receptors in hGRK4γ142A>V transgenic mice, the vehicle (saline) or recombinant mouse adiponectin (0.5–5 μg/kg/min) was infused into the external jugular vein. Urine was collected via a suprapubic cystostomy, as previously reported, with modification [13]. Urinary sodium concentration was measured with an electrolyte analyzer (HC988, Histrong Medical, Shenzhen, China), using an ion-selective electrode.

Ultrasound-targeted microbubble destruction treatment

The ultrasound-targeted microbubble destruction (UTMD)-mediated GRK4 small interfering RNA (siRNA) was delivered into kidney, as reported [16]. Seven-week-old SHRs were fed *ad libitum* for 1 week before the treatment of UTMD. Briefly, the rats were placed in the left lateral position after anesthesia by the intraperitoneal injection of 2% pentobarbital sodium at a dose of 50 mg/kg. The right kidney was located and irradiated using the 9L4 linear array probe of an ultrasound imaging system for 5 min of continuous irradiation (42 frames per second, mechanical index = 0.9, frequency = 7.00 MHz). The probe was positioned in a transducer holder at an appropriate place to treat right kidney, and then, the contralateral

kidney was treated in the same way, by UTMD. Microbubbles in the injectate were generated by shaking for 90 s in a custom-built oscillator. The microbubbles carrying GRK4 siRNA and scramble siRNA were injected into the lateral tail vein through a 26-gauge needle connected to a 1-ml syringe via a 15-cm-long catheter (0.45915 round wall, long bevel, China), controlled by a syringe pump. The injection was completed within 60 s and 0.5 ml saline was used to flush the tube. The UTMD treatments were performed every 3 days. Each rat received a total of ten-times of UTMD treatments. The rats were 12 weeks old after 4 weeks UTMD treatments.

All procedures in the present study were approved by the Third Military Medical University Animal Use and Care Committee. All efforts were made to minimize animal suffering and reduce the number of animals used.

Cell culture

Immortalized renal proximal tubule (RPT) cells from WKY rats and SHRs were cultured at 37°C in 95% air and 5% CO_2. The RPT cells were grown in DMEM/F12 with insulin, transferrin, epidermal growth factor, and dexamethasone [10,11,16]. The RPT cells (95% confluence) were serum-deprived for 2 h, and then treated with vehicle, adiponectin (Biovision), Compound C (adenosine $5'$-monophosphate (AMP)-activated protein kinase (AMPK) antagonist, 10^{-7} mol/l), NG-nitro-L-arginine methyl ester (L-NAME; non-selective NOS inhibitor, 5×10^{-7} mol/l) (Sigma–Aldrich), alone, or in combination. The inhibitors were added 30 min before the addition of adiponectin. Then, the treated cells were extracted in cold cell lysis buffer, sonicated, kept on ice for 1 h, then centrifuged at the speed of 14000 $\times g$ for 30 min to collect the supernatant.

Immunoblotting

The proteins in the supernatant of the centrifuged samples were separated by SDS/PAGE and transferred on to nitrocellulose membranes after blocking in skim milk for 1 h. The blots were then incubated with the indicated anti-bodies: rabbit anti-rat adiponectin, rabbit anti-rat adiponectin receptor 1 ($AdipoR_1$; 1:1000; Cell Signaling, Davers, MA), mouse anti-rat adiponectin receptor 2 ($AdipoR_2$; 1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-rat phosphorylated AMPK, rabbit anti-rat AMPK, rabbit anti-rat phospho-AMPK (1:1000; Cell Signaling), and rabbit anti-rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:500; Millipore, Darmstadt, Germany). The specificity of the antibody was determined by mixing the blocking peptide with the corresponding antibody at a ratio of 10:1 overnight in an oscillator in 4°C, and then incubated with IRDye secondary antibody (Li-COR, Lincoln, NE). The bands were detected by the Odyssey Infrared Imaging System (Li-COR). The densities of the bands were semi-quantified and analyzed by the Odyssey Application Software (Li-COR). The amount of protein transferred on ghnto the membranes was verified by immunoblotting for GAPDH.

Immunoprecipitation

Equal amounts of tissue lysates (1000 μg protein/ml supernatant) were immunoprecipitated with antibody against $AdipoR_1$ or $AdipoR_2$ (3 μl/ml) for 1 h and protein-G agarose at 4°C for 12 h. The immunoprecipitates were subjected to immunoblotting with mouse anti-rat

Gαi antibody (Santa Cruz Biotechnology Inc.). To determine the specificity of the bands found on the immunoblots, IgG (negative control) and Gαi antibodies (positive control) were used as the immunoprecipitants, instead of the $AdipoR_1$ (or $AdipoR_2$) antibody. To determine the phosphorylation of the $AdipoR_1$ (or $AdipoR_2$), the supernatants were immunoprecipitated with anti-$AdipoR_1$ (or anti-$AdipoR_2$) antibody. Then, the immunoprecipitates were subjected to immunoblotting with the anti-phosphoserine or anti-phosphothreonine antibodies (Santa Cruz Biotechnology Inc.).

Na^+-K^+-ATPase activity assay

Na^+-K^+-ATPase activity assay was performed as described in our previous reports [10–12]. After washing twice with chilled phosphate-free buffer, the pellet (membrane fraction) was suspended in 500 μl of 10 mM Tris-HCl and 1 mM EDTA (pH 7.5) on ice. The reactions were initiated by adding ATP (4 mM) and terminated after 15 min of incubation at 37°C by adding 50 μl of 50% trichloracetate. Na^+-K^+-ATPase activity was the difference between total and ouabain-insensitive ATPase activity, and expressed as nmol phosphate released per mg protein per min.

Transfection of AdipoR siRNA into RPT cells

The siRNA (100 nmol) targeting *$AdipoR_1$* or *$AdipoR_2$* was pre-incubated with Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM® medium (Gibco, CarIsbad, CA) for 20 min, and then added the mixture into RPT cells (60–70% confluent), according to the manufacturer's instructions. The cells were then incubated in 0.1% FBS containing DMEM for an additional 24 or 48 h before each experiment. The expression of AdipoRs was detected by real-time quantitative PCR (qRT-PCR) and immunoblotting. The rat gene-specific siRNAs are shown in Supplementary Table S1.

Transfection of AdipoR plasmid into RPT cells

Rat $AdipoR_1$ plasmid WT and mutant with Ser^7, Thr^{24}, Thr^{43} replaced by nonphosphorylatable alanines (Ala) were prepared. The plasmid was pre-incubated with Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM® medium (Gibco, Carlsbad, CA) for 20 min, and then added the mixture to the 60–70% confluent RPT cells (final concentration, 2 μg/ml). The cells were then incubated in 0.1% FBS, containing DMEM, for an additional 24 or 48 h before each experiment. The expression of $AdipoR_1$ was detected by qRT-PCR and immunoblotting. We found that $AdipoR_1$ was the major adiponectin receptor desensitized in the SHR (see below). Therefore, we concentrated our studies on $AdipoR_1$.

Real-time quantitative PCR

Total RNA, isolated using TRIzol reagent, was quantified spectrophotometrically (DU800, Beckman Coulter, Brea, CA). Two micrograms of total RNA extracted from RPT cells were used to synthesize cDNA as a template for amplification of $AdipoR_1$ and $AdipoR_2$; GAPDH served as the housekeeping gene control (Invitrogen). qRT-PCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). For amplification, 2 μl of cDNA were used per 25 μl final reaction volume. Polymerase chain reaction conditions included initial denaturation at 95°C for 3 min, followed by 39 cycles of denaturation at

95°C for 10 s, and annealing at 62°C for 30 s. Quantification cycle (C_q) values were analyzed automatically using the Bio-Rad CFX manager 2.1 (Bio-Rad). $AdipoR_1$ and $AdipoR_2$ RNA expressions were normalized by GAPDH. The rat gene-specific primers used for PCR are shown in Supplementary Table S2.

Immunofluorescence

The kidneys form WKY rats were paraffin-embedded and dehydrated with ethanol. The sections were incubated with 3% H_2O_2 for 10 min, and then incubated with 1% albumin bovine serum (Gibco) for 30 min at room temperature. RPT cells on coverslips, in a 24-well plate, were fixed with 4% paraformaldehyde and then incubated with 10 mM citrate buffer (pH 6.0) twice for 5 min. $AdipoR_1$ was immunostained with rabbit anti-rat $AdipoR_1$ antibody, followed by Alexa Fluor 488 goat anti-rabbit antibody (Molecular Probes, OR). $AdipoR_2$ was immunostained with mouse anti-rat $AdipoR_2$ antibody, followed by Cy3-labeled donkey anti-mouse antibody (Beyotime Institute of Biotechnology, Haimen, China). The anti-$AdipoR_1$ and anti-$AdipoR_2$ antibodies (1:100) were incubated at 4°C overnight. Secondary antibody was added and then the slides were kept at room temperature for 60 min. After DAPI staining for nuclei, the sections and coverslips were photographed (DN100, E600; Nikon Co., Tokyo, Japan).

Statistical analysis

The data are expressed as mean ± SEM. Comparison within groups was made by repeated-measures ANOVA (or two-tailed paired *t* test when only two groups were compared); comparison among groups was made by one-way factorial ANOVA with Holm–Sidak's test (or unpaired *t* test when only two groups were compared). $P<0.05$ was considered significant.

Results

Diuretic and natriuretic effects of adiponectin in WKY rats and Adipo$^{-/-}$ mice

To determine the effect of adiponectin on renal function, we first studied the diuresis and natriuresis in adiponectin WT (Adipo$^{+/+}$) and Adipo$^{-/-}$ mice; their general characteristics are shown in Supplementary Figure S1. Compared with Adipo$^{+/+}$ mice, Adipo$^{-/-}$ mice (Supplementary Figure S1A,B) had increased body weight, visceral fat, and blood pressure (Supplementary Figure S1C–F). The 24-h water and sodium excretion were reduced in Adipo$^{-/-}$ mice, relative to Adipo$^{+/+}$ mice (Figure 1A,B), indicating that adiponectin has diuretic and natriuretic effects.

Diuretic and natriuretic effects of adiponectin were further confirmed in WKY rats. WKY rats were infused with recombinant adiponectin (0.1,1.0,10.0 μg/kg per minute for 40 min in each period), via the right suprarenal artery. Results showed that adiponectin increased urine flow (UV) and absolute sodium excretion (UNaV) in a dose-dependent manner (Figure 1C). It should be noted that infusion of adiponectin via suprarenal artery, that increased sodium excretion, was not associated with a change in blood pressure (Supplementary Figure S1F). As a control, boiled adiponectin, instead of recombinant adiponectin, was infused in the

same manner. Boiled adiponectin had no effect on either water and sodium excretion or blood pressure (Figure 1C and Supplementary Figure S1F).

The expression of adiponectin receptor was studied in the kidney. Two subtypes of adiponectin receptors, $AdipoR_1$ and $AdipoR_2$, were expressed in all nephron segments, including the RPT, at the apical and basolateral membranes (Figure 1D), and in immortalized rat RPT cells (Figure 1E). The $AdipoR_1$ protein (≈43 kDa) band and $AdipoR_2$ protein (≈44 kDa) band in the kidney and RPT cells were no longer visible when the antibodies were pre-adsorbed by the immunizing peptide (Supplementary Figure S2A).

Impaired natriuretic and diuretic effects of adiponectin in hypertension

To determine the effect of adiponectin in hypertension, same dosages of adiponectin (0.1, 1.0, 5.0 μg/kg per minute for 40 min in each period) were infused via the right suprarenal artery in younger (4 weeks old) and older (12 weeks old) rats. And the body weight and blood pressure of WKY and SHR rats at different ages are shown in Supplementary Figure S3. We found that adiponectin-mediated natriuresis and diuresis were markedly impaired in either younger or older SHRs (Figure 2A,B). Impaired sodium transport effect of adiponectin was also confirmed in *in vitro* experiment, i.e., Na^+-K^+-ATPase activity in RPT cells. Adiponectin inhibited Na^+-K^+-ATPase activity in a concentration- and time-dependent manner in RPT cells from WKY rats (Figure 2C and Supplementary Figure S2B), while the inhibitory effect of adiponectin on Na^+-K^+-ATPase activity was impaired in RPT cells from SHRs (Figure 2D).

To explore the possible mechanisms underlying impaired adiponectin effects in hypertension, we first checked serum adiponectin level and renal adiponectin receptor expression in SHRs. Serum adiponectin levels were lower in SHRs than those in WKY rats (Supplementary Figure S4A). However, there were no differences in $AdipoR_1$ and $AdipoR_2$ protein expressions in the renal cortex from those two strains (Supplementary Figure S4B,C). The lost natriuretic and diuretic effects of adiponectin could not be explained by the decreased serum adiponectin level in SHRs, because the serum adiponectin level in SHRs was only approximately half those of WKY rats. Even with increased dosage of adiponectin from 0.1 to 1.0 μg/kg/min, impaired adiponectin-mediated diuresis and natriuresis were still apparent in SHRs (Figure 2A).

Hyperphosphorylation of adiponectin receptors and its uncoupling from Gαi may lead to adiponectin dysfunction in hypertension

A previous study showed that phosphorylation desensitizes $AdipoR_1$ in post-myocardial infarction failing heart [17]. Our present study showed that the phosphorylation of $AdipoR_1$ and $AdipoR_2$ at both serine and threonine residues were increased in the kidneys from SHRs, as compared with those from WKY rats (Figure 3A,B). It should be noted that relative to WKY rats, the phosphorylation of $AdipoR_1$ in SHRs was increased two- to three-fold, while the phosphorylation of $AdipoR_2$ was only increased by 20–50%, suggesting that desensitized $AdipoR_1$ played the main role in the SHRs. Similar observations were made with younger SHRs (4-week-old) (Supplementary Figure S5A,B). Therefore, in subsequent experiments, we focused on $AdipoR_1$, rather than $AdipoR_2$.

To determine further the contribution of $AdipoR_1$ phosphorylation on the $AdipoR_1$ dysfunction, we analyzed $AdipoR_1$ using bioinformatics analysis (website, http://gps.biocuckoo.org/online.php), and found three phosphorylation sites (Ser^7/Thr^{24}/Thr^{43}) (Supplementary Figure S6A,B). After mutation of those sites (Ser^7, Thr^{24}, and Thr^{43} replaced by Ala) and transfection of those mutation plasmid in SHR RPT cells (Supplementary Figure S6C,D), we found that the mutation of the $AdipoR_1$ phosphorylation sites reduced $AdipoR_1$ phosphorylation (Figure 3C), and rescued impaired adiponectin-mediated inhibition of Na^+-K^+-ATPase activity in SHR RPT cells (Figure 3D), indicating that the hyperphosphorylation of $AdipoR_1$ was the key step in the $AdipoR_1$ dysfunction in kidney in hypertension.

Previous studies showed that persistent receptor phosphorylation leads to the uncoupling of G protein-coupled receptors (GPCRs) from their associated G proteins in kidney, which leads to the GPCR dysfunction [13,18–22]. Although distinct from GPCRs, progestin and adipoQ (adiponectin) receptors (PAQRs), to which AdipoR belongs, can be transduced by G proteins to activate downstream signaling [23]. Our present study found a coupling between $AdipoR_1$ and Gαi (Figure 4A) but not with Gαq and Gαs (Supplementary Figure S7A,B) in kidneys from WKY rats. In the presence of pertussis toxin (PTX), a Gαi inhibitor [24–26], the adiponectin-mediated AMPK phosphorylation, a downstream signaling of adiponectin, was completely blocked (Figure 4B), and consequently, the inhibitory effect of adiponectin on Na^+-K^+-ATPase activity was lost (Figure 4C), indicating the importance of Gαi in the $AdipoR_1$ signaling. We further found that, consistent with impaired $AdipoR_1$ function, the $AdipoR_1$/Gαi coupling was reduced in SHRs, determined by co-immunoprecipitation (Figure 4A); consistent with the recovery of adiponectin-mediated inhibition of Na^+-K^+-ATPase activity after mutation of the $AdipoR_1$ phosphorylation sites in SHR RPT cells (Figure 3D). Mutation of the $AdipoR_1$ phosphorylation sites also restored $AdipoR_1$ and Gαi coupling in SHR cells (Figure 4D), which indicated that $AdipoR_1$ and Gαi uncoupling is one vital step leading to the impaired natriuretic effect of adiponectin in hypertension.

GRK4 leads to hyperphosphorylation and impairment of adiponectin receptor function in hypertension

The phosphorylation of adiponectin receptor is regulated by GRK2 in the heart [17]. Because GRK4 is an important GRK subtype in the kidney, and increased renal GRK4 expression or activity by GRK4 gene variants (e.g., 65R>L, 142A>V, and 486A>V) hyperphosphorylates dopamine receptors [13,19,20], we wondered whether GRK4 can also hyperphosphorylate adiponectin receptors. First, we studied the renal effect of adiponectin in GRK4γ142A>V transgenic mice, which have hypertension and impaired sodium excretion [13,15,19,20], to see whether GRK4γ transgenic mice could replicate the abnormal adiponectin function in SHRs. We found that the natriuresis and diuresis induced by adiponectin were lesser in GRK4γ142A>V mice than GRK4γWT mice (Figure 5A,B). There was higher phosphorylation of $AdipoR_1$ in kidney from GRK4γ142A>V mice; the linkage between $AdipoR_1$ and Gαi was reduced in GRK4γ142A>V mice as compared with GRK4γWT mice (Figure 5C,D), indicating that GRK4 is involved in the adiponectin receptor dysfunction in hypertension.

To further study the role of GRK4 in the regulation of adiponectin receptor function, we studied the effect of down-regulating GRK4 expression on adiponectin receptor function and blood pressure in SHRs, whose GRK4 expression has been reported to be increased [27]. Renal GRK4 expression was down-regulated via UTMD-mediated GRK4 siRNA (1 OD per time) into the kidney (Supplementary Figure S8A–C). The UTMD treatments were performed every 3 days. Each rat received a total of tentreatments. Four weeks of GRK4 siRNA-UTMD treatment decreased the blood pressure in SHRs (Figure 6A), accompanied by an increase in water and sodium excretion (Figure 6B,C). Impaired adiponectin-mediated water and sodium excretion recovered to some degree (Figure 6D,E). The phosphorylation of $AdipoR_1$ at both serine and threonine residues were reduced (Figure 6F,G), and the coupling between $AdipoR_1$ and Gαi was increased after GRK4 siRNA-UTMD treatment (Figure 6H).

Discussion

Clinical studies have shown an inverse relationship between plasma adiponectin and blood pressure; hypertensive patients have decreased adiponectin levels, especially in those with visceral fat accumulation [4,5,28]. Deletion of the Adipo gene does not alter blood pressure in 3-month-old or younger mice [29,30]. Our present study also shows a slightly but not significantly greater blood pressure of $Adipo^{-/-}$ mice than $Adipo^{+/+}$ mice at 4 months of age. However, the blood pressures of $Adipo^{-/-}$ mice progressively increased from 5 to 7 months of age. A high salt diet (8% NaCl) also increases blood pressure in 2–2.5 month-old $Adipo^{-/-}$ mice that is ameliorated by adenovirus-mediated re-expression of adiponectin [6]. Therefore, we focused on adiponectin function in the kidney in the present study. Adiponectin receptors include $AdipoR_1$ and $AdipoR_2$, both of which are found in the kidney, especially in the RPT cells in the present study.

The AdipoRs have been once classified as PAQR [23]. The sequence homology between PAQR and the classic members of GPCR families is low, and is structurally, topologically, and functionally distinct from classic GPCRs [23,31]. Nevertheless, PAQRs 5,7,8 (membrane progesterone receptor γ, α, β) were predicted to interact with G proteins [32]. The PAQR's inhibitory effect on cAMP production is abolished by PTX, an inhibitor of Gαi protein [32]. Our present study confirms that AdipoRs are functionally coupled to Gαi, because adiponectin-induced AMPK activation by AdipoRs is almost completely inhibited by PTX.

To determine a role of adiponectin on sodium excretion, we infused adiponectin, selectively into the right kidney, via the right suprarenal artery. We found that adiponectin causes a dose-related diuresis and natriuresis in WKY rats. The diuretic and natriuretic effect is not secondary to an increase in blood pressure, because we did not find any change in blood pressure during the intrarenal infusion of adiponectin, via the suprarenal artery in WKY rats. Furthermore, our present study showed that diuretic and natriuretic effects of adiponectin are markedly impaired in SHRs. The reduced adiponectin receptor function in SHRs could be ascribed to several mechanisms. Consistent with a previous report [30], the plasma adiponectin level is lower in SHRs than WKY rats. However, even with higher concentration of adiponectin, the inhibitory effect of adiponectin on renal Na^+-K^+-ATPase activity could

not be achieved, indicating that in addition to decreased adiponectin concentration, other mechanisms play a more important role in the adiponectin dysfunction in hypertension. Therefore, we focused on the changes in adiponectin receptor expression and function in hypertension. Our finding showed that diuretic and natriuretic effects of adiponectin are markedly impaired in SHRs, which is inconsistent with the report of Afzal et al. [33]. They found that the intraperitoneal injection of adiponectin (2.5 μg/kg/day for 7 days) increased urine flow and sodium excretion in SHRs. Although the reasons leading to the differences are not clear, the abnormal function of adiponectin in the kidney of SHRs maybe age-related or affected by the route of administration. Our studies were performed in 3- to 4-month-old SHRs, in which adiponectin was infused selectively into the kidney. Afzal et al. did not indicate the age of the rats but their weights (230–255 grams) are those reported for 9- to 10-week-old SHRs. Moreover, the present study treated the rats for 7 days; the long-term treatment with adiponectin can negatively affect the sympathetic and renin–angiotensin systems [34,35].

Phosphorylation of GPCRsis an important step in receptor signaling [18–21,36]. However, prolonged GPCR phosphorylation impairs its function, as has been reported for dopamine D_1 receptor [13,19,20]. Our present study found that renal $AdipoR_1$ is hyperphosphorylated and functionally impaired in SHRs; mutation of the adiponectin receptor phosphorylation sites reduces its phosphorylation and restores the coupling of $AdipoR_1$ and Gαi and the ability of adiponectin to inhibit Na^+-K^+-ATPase activity. The phosphorylation of GPCR is regulated by GRKs [13–21,27,36]. Among the seven GRK subtypes, GRK4 is distinguished from other members of the GRK family by its constitutive activity and limited tissue expression [13,19,20]. The GRK4 variants 65L, 142V, and 486V are associated with essential hypertension in ethnically distinct populations [19,20]. Overexpression of human(h) GRK4γ142A>V or hGRK4γ486A>V in mice produces hypertension [13,15,37]. Therefore, GRK4 plays an important role in the regulation of blood pressure and in the pathogenesis of hypertension [13–16,19,20]. It is reported that GRK2 is important in the regulation of AdipoR function [38,]. AdipoRs are phosphorylated by GRK2 in cardiomyocytes during post-myocardial infarction remodeling and heart failure [17,]. Whether or not GRK4 regulates AdipoR is not known. We now report that adiponectin receptor can be regulated by GRK4, as shown in hGRK4γ142A>V transgenic mice. The adiponectin receptor dysfunction in SHRs is completely replicated in hGRK4γ142A>V transgenic mice, i.e., absence of adiponectin-mediated diuresis and natriuresis, hyperphosphorylation of $AdipoR_1$ and uncoupling of $AdipoR_1$ from Gαi. To prove that renal GRK4 is important in the adiponectin receptor phosphorylation and impaired function in the SHR, we silenced GRK4 by UTMD-mediated siRNA in the kidneys of SHRs. This renal-restricted silencing of GRK4 lowers the blood pressure of SHRs and increases sodium excretion. This is associated with a decrease in adiponectin receptor phosphorylation, and restoration of the uncoupling between $AdipoR_1$ and Gαi, and adiponectin-stimulated sodium excretion.

In conclusion, we demonstrated that adiponectin is an important link between the adipose tissue and kidney and plays an important role in increasing sodium and water excretion. The stimulatory effect of adiponectin on sodium excretion is impaired in hypertension, which can be ascribed to the increased renal GRK4 expression and activity. Renal-specific down-

regulation of GRK4 in hypertension allows the recovery of adiponectin-mediated sodium excretion and consequently lowers the blood pressure in spontaneous hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Adipo$^{-/-}$	adiponectin knockout
AdipoR$_1$	adiponectin receptor 1
AdipoR$_2$	adiponectin receptor2
AMPK	adenosine 5′-monophosphate (AMP)-activated protein kinase
DAPI, 4’	6-diamidino-2-phenylindole
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
GRK4	G protein-coupled receptor kinase 4
PAQR	progestin and adipoQ (adiponectin) receptor
PTX	pertussis toxin
RPT	renal proximal tubule
SHR	spontaneously hypertensive rat
siRNA	small interfering RNA
WKY	Wistar–Kyoto

References

1. Lawes CM, Vander Hoorn S, Rodgers A and International Society of Hypertension (2008) Global burden of blood-pressure-related disease, 2001. Lancet 371, 1513–1518, 10.1016/S0140-6736(08)60655-8 [PubMed: 18456100]
2. Cao H (2014) Adipocytokines in obesity and metabolic disease. J. Endocrinol 220, 47–59, 10.1530/J0E-13-0339

3. Hall JE, do Carmo JM, da Silva AA, Wang Z and Hall ME (2019) Obesity, kidney dysfunction and hypertension: mechanistic links. Nat. Rev. Nephrol 15, 367–385, 10.1038/s41581-019-0145-4 [PubMed: 31015582]
4. Yamauchi T and Kadowaki T (2013) Adiponectin receptor as a key player in healthy longevity and obesity-related diseases. Cell Metab. 17, 185–196, 10.1016/j.cmet.2013.01.001 [PubMed: 23352188]
5. Iwashima Y, Katsuya T, Ishikawa K et al. (2004) Hypoadiponectinemia is an independent risk factor for hypertension. Hypertension 43, 1318–1323, 10.1161/01.HYP.0000129281.03801.4b [PubMed: 15123570]
6. Ohashi K, Kihara S, Ouchi N, Kumada M, Fujita K, Hiuge A et al. (2006) Adiponectin replenishment ameliorates obesity-related hypertension. Hypertension 47, 1108–1116, 10.1161/01.HYP.0000222368.43759.a1 [PubMed: 16651465]
7. Coffman TM and Crowley SD (2008) Kidney in hypertension: Guyton redux. Hypertension 51, 811–816, 10.1161/HYPERTENSIONAHA.105.063636 [PubMed: 18332286]
8. Horita S, Seki G, Yamada H, Suzuki M, Koike K and Fujita T (2013) Roles of renal proximal tubule transport in the pathogenesis of hypertension. Curr. Hypertens. Rev 9, 148–155, 10.2174/15734021113099990009 [PubMed: 23971696]
9. Brands MW (2012) Chronic blood pressure control. Compr. Physiol 2, 2481–2494 [PubMed: 23720255]
10. Chen Y, Asico LD, Zheng S, Villar VA, He D, Zhou L et al. (2013) Gastrin and D1 dopamine receptor interact to induce natriuresis and diuresis. Hypertension 62, 927–933, 10.1161/HYPERTENSIONAHA.113.01094 [PubMed: 24019399]
11. Yang S, Han Y, Zheng S, Kou X, Asico LD, Huang H et al. (2015) Enhanced natriuresis and diuresis in Wistar rats caused by the costimulation of renal dopamine D3 and angiotensin II type 2 receptors. Am. J. Hypertens 28, 1267–1276, 10.1093/ajh/hpv018 [PubMed: 25770092]
12. Luo H, Chen C, Guo L, Xu Z, Peng X, Wang X et al. (2018) Exposure to maternal diabetes mellitus causes renal dopamine D1 receptor dysfunction and hypertension in adult rat offspring. Hypertension 72, 962–970, 10.1161/HYPERTENSIONAHA.118.10908 [PubMed: 30354705]
13. Felder RA, Sanada H, Xu J, Yu PY, Wang Z, Watanabe H et al. (2002) G protein-coupled receptor kinase 4 gene variants in human essential hypertension. Proc. Natl. Acad. Sci. U.S.A 99, 3872–3877, 10.1073/pnas.062694599 [PubMed: 11904438]
14. Chen K, Fu C, Chen C, Liu L, Ren H, Han Y et al. (2014) Role of GRK4 in the regulation of arterial AT1 receptor in hypertension. Hypertension 63, 289–296, 10.1161/HYPERTENSIONAHA.113.01766 [PubMed: 24218433]
15. Wang Z, Zeng C, Villar VA, Chen SY, Konkalmatt P, Wang X et al. (2016) Human GRK4γ142V variant promotes angiotensin II type I receptor-mediated hypertension via renal histone deacetylase type 1 inhibition. Hypertension 67, 325, 10.1161/HYPERTENSIONAHA.115.05962 [PubMed: 26667412]
16. Huang H, Li X, Zheng S, Chen Y, Chen C, Wang J et al. (2016) Downregulation of renal G protein-coupled receptor kinase type 4 expression via ultrasound-targeted microbubble destruction lowers blood pressure in spontaneously hypertensive rats. J. Am. Heart Assoc 5, e004028, 10.1161/JAHA.116.004028 [PubMed: 27792639]
17. Wang Y, Gao E, Lau WB, Wang Y, Liu G, Li JJ et al. (2015) G-protein–coupled receptor kinase 2–mediated desensitization of adiponectin receptor 1 in failing heart. Circulation 131,1392–1404, 10.1161/CIRCULATIONAHA.114.015248 [PubMed: 25696921]
18. Brinks HL and Eckhart AD (2010) Regulation of GPCR signaling in hypertension. Biochim. Biophys. Acta 1802,1268–1275, 10.1016/j.bbadis.2010.01.005 [PubMed: 20060896]
19. Yang J, Villar VA, Jones JE, Jose PA and Zeng C (2015) G protein-coupled receptor kinase 4: role in hypertension. Hypertension 65, 1148–1155, 10.1161/HYPERTENSIONAHA.115.05189 [PubMed: 25870190]
20. Yang J, Villar VA, Armando I, Jose PA and Zeng C (2016) G protein-coupled receptor kinases: crucial regulators of blood pressure. J. Am. Heart Assoc 5, pii: e003519, 10.1161/JAHA.116.003519 [PubMed: 27390269]

21. Tobin AB (2008) G-protein-coupled receptor phosphorylation: where, when and by whom. Br. J. Pharmacol 153, S167–S176, 10.1038/sj.bjp.0707662 [PubMed: 18193069]
22. Raake PW, Vinge LE, Gao E, Boucher M, Rengo G, Chen X et al. (2008) G protein-coupled receptor kinase 2 ablation in cardiac myocytes before or after myocardial infarction prevents heart failure. Circ. Res 103, 413–422, 10.1161/CIRCRESAHA.107.168336 [PubMed: 18635825]
23. Tang YT, Hu T, Arterburn M, Boyle B, Bright JM, Emtage PC et al. (2005) PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. J. Mol. Evol 61, 372–380, 10.1007/s00239-004-0375-2 [PubMed: 16044242]
24. Yue P, Jin H, Xu S, Aillaud M, Deng AC, Azuma J et al. (2011) Apelin decreases lipolysis via G(q), G(i), and AMPK-dependent mechanisms. Endocrinology 152, 59–68, 10.1210/en.2010-0576 [PubMed: 21047945]
25. Kopp C, Singh SP, Regenhard P, Müller U, Sauerwein H and Mielenz M (2014) Trans-cinnamic acid increases adiponectin and the phosphorylation of AMP-activated protein kinase through G-protein-coupled receptor signaling in 3T3–L1 adipocytes. Int. J. Mol. Sci 15, 2906–2915, 10.3390/ijms15022906 [PubMed: 24557583]
26. Lu J, Fang B, Zheng Y, Yu X, Huang G, Wang Z et al. (2017) 1,3-dichloro-2-propanol induced lipid accumulation in HepG2 cells through cAMP/protein kinase A and AMP-activated protein kinase pathways via Gi/o-coupled receptors. Environ. Toxicol. Pharmacol 55,118–126, 10.1016/j.etap.2017.07.013 [PubMed: 28843851]
27. Sanada H, Yatabe J, Midorikawa S, Katoh T, Hashimoto S, Watanabe T et al. (2006) Amelioration of genetic hypertension by suppression of renal G protein-coupled receptor kinase type 4 expression. Hypertension 47, 1131–1139, 10.1161/01.HYP.0000222004.74872.17 [PubMed: 16636192]
28. Adamczak M, Wiecek A, Funahashi T, Chudek J, Kokot F and Matsuzawa Y (2003) Decreased plasma adiponectin concentration in patients with essential hypertension. Am. J. Hypeitens 16, 72–75, 10.1016/S0895-7061(02)03197-7
29. Qi GM, Jia LX, Li YL, Li HH and Du J (2014) Adiponectin suppresses angiotensin II-induced inflammation and cardiac fibrosis through activation of macrophage autophagy. Endocrinology 155, 2254–2265, 10.1210/en.2013-2011 [PubMed: 24684303]
30. Xing W, Yan W, Liu P, Ji L, Li Y, Sun L et al. (2013) A novel mechanism for vascular insulin resistance in normotensive young SHRs: hypoadiponectinemia and resultant APPL1 downregulation. Hypertension 61,1028–1035, 10.1161/HYPERTENSIONAHA.111.00728 [PubMed: 23478100]
31. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S et al. (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423, 762–769, 10.1038/nature01705 [PubMed: 12802337]
32. Zhu Y, Rice CD, Pang Y, Pace M and Thomas P (2003) Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. U.S.A 100, 2231–2236, 10.1073/pnas.0336132100 [PubMed: 12574519]
33. Afzal S, Sattar MA, Johns EJ, Abdulla MH, Akhtar S, Hashmi F et al. (2016) Interaction between irbesartan, peroxisome proliferator-activated receptor (PPAR-γ), and adiponectin in the regulation of blood pressure and renal function in spontaneously hypertensive rats. J. Physiol. Biochem 72, 593–604, 10.1007/s13105-016-0497-1 [PubMed: 27405250]
34. Allison MA, Jenny NS, McClelland RL, Cushman M and Rifkin D (2015) The associations of adipokines with selected markers of the renin-angiotensinogen-aldosterone system: the multi-ethnic study of atherosclerosis. J. Hum. Hypertens 29, 127–133, 10.1038/jhh.2014.40 [PubMed: 24919752]
35. Canale MP, Manca di Villahermosa S, Martino G, Rovella V, Noce A, De Lorenzo A et al. (2013) Obesity-related metabolic syndrome: mechanisms of sympathetic overactivity. Int. J. Endocrinol 2013, 865965, 10.1155/2013/865965 [PubMed: 24288531]
36. Gurevich VV and Gurevich EV (2019) GPCR signaling regulation: the role of GRKs and arrestins. Front. Pharmacol 10, 125, 10.3389/fphar.2019.00125 [PubMed: 30837883]

37. Diao Z, Asico LD, Villar VAM, Zheng X, Cuevas S, Armando I et al. (2017) Increased renal oxidative stress in salt-sensitive human GRK4γ486V transgenic mice. Free Radic. Biol. Med 106, 80–90, 10.1016/j.freeradbiomed.2017.02.021 [PubMed: 28189851]

38. Murga C, Arcones AC, Cruces-Sande M, Briones AM, Salaices M and Mayor F Jr (2019) G protein-coupled receptor kinase 2 (GRK2) as a pot.ential therapeutic target in cardiovascular and metabolic diseases. Front. Pharmacol 10, 112, 10.3389/fphar.2019.00112 [PubMed: 30837878]

Clinical perspectives

- Whether and how adiponectin, one of the adipokines, contributes to impaired sodium excretion in hypertension has not been previously investigated.
- We conclude that the stimulatory effect of adiponectin on sodium excretion is impaired in hypertension, which is ascribed to the increased renal GRK4 expression and activity.
- Targeting GRK4 restores impaired adiponectin-mediated sodium excretion in hypertension, thus representing a novel strategy against hypertension.

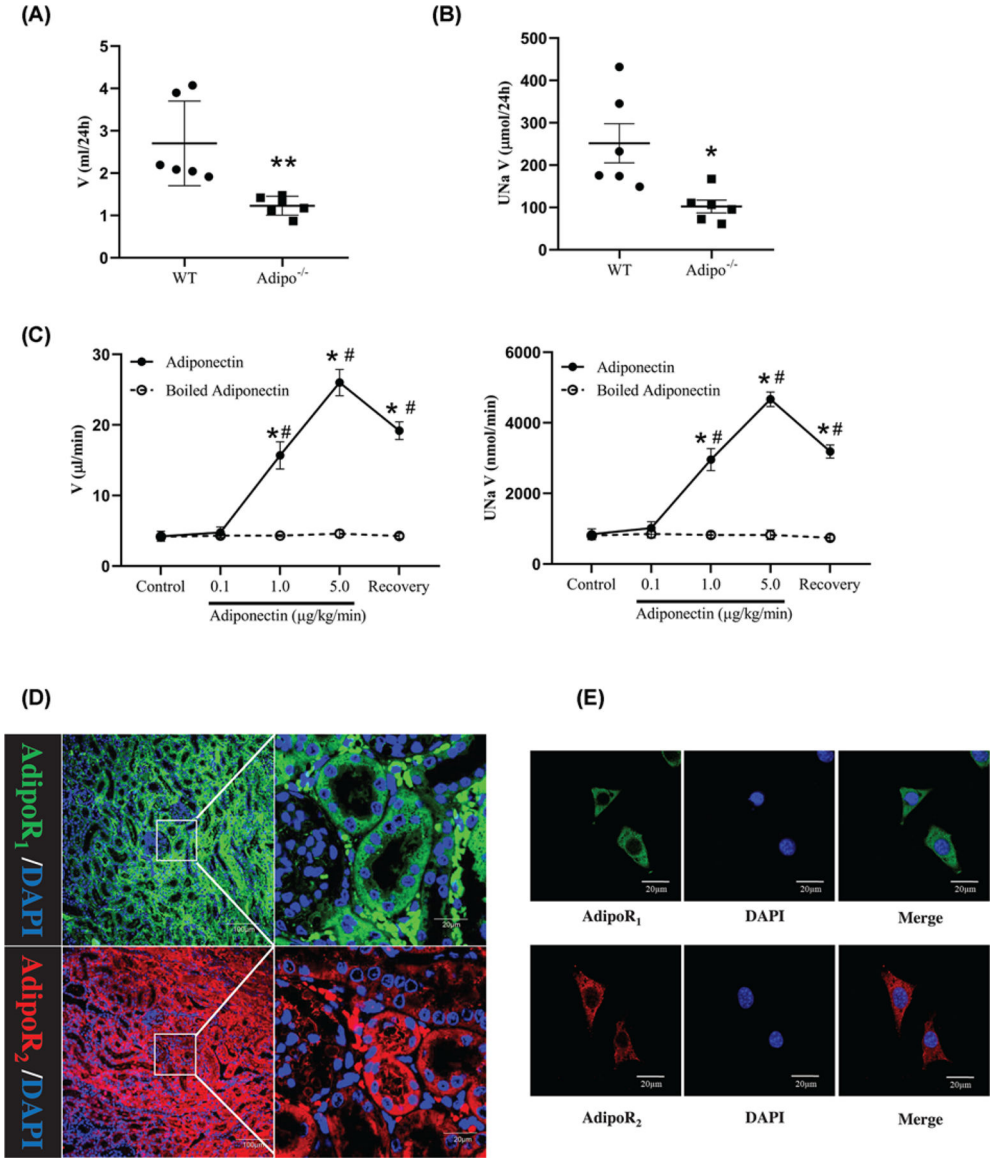

Figure 1. Diuretic and natriuretic effects of adiponectin in WKY rats and Adipo$^{-/-}$

(**A,B**) Decreased 24-h urine volume and urinary sodium excretion in 4-month-old Adipo$^{-/-}$ mice. Urine samples were collected for 24 h in metabolic cages (**P<0.01 vs. WT, *P<0.05 vs. WT, n=6/group). (**C**) Increasing doses of adiponectin or boiled adiponectin (0.1–5.0 μg/kg per min) were infused into the right renal artery, via the right suprarenal artery, of 12-week-old WKY rats. The renal function was determined by urine flow (V) and sodium excretion (UNaV) (*P<0.05 vs. boiled adiponectin; $^{\#}P$<0.05 vs. control, n=4). (**D,E**) Immunofluorescence of AdipoR$_1$ and AdipoR$_2$ in renal cortex and RPT cells from WKY rats. AdipoR$_1$ was labeled green, AdipoR$_2$ was labeled red, and DAPI was labeled blue.

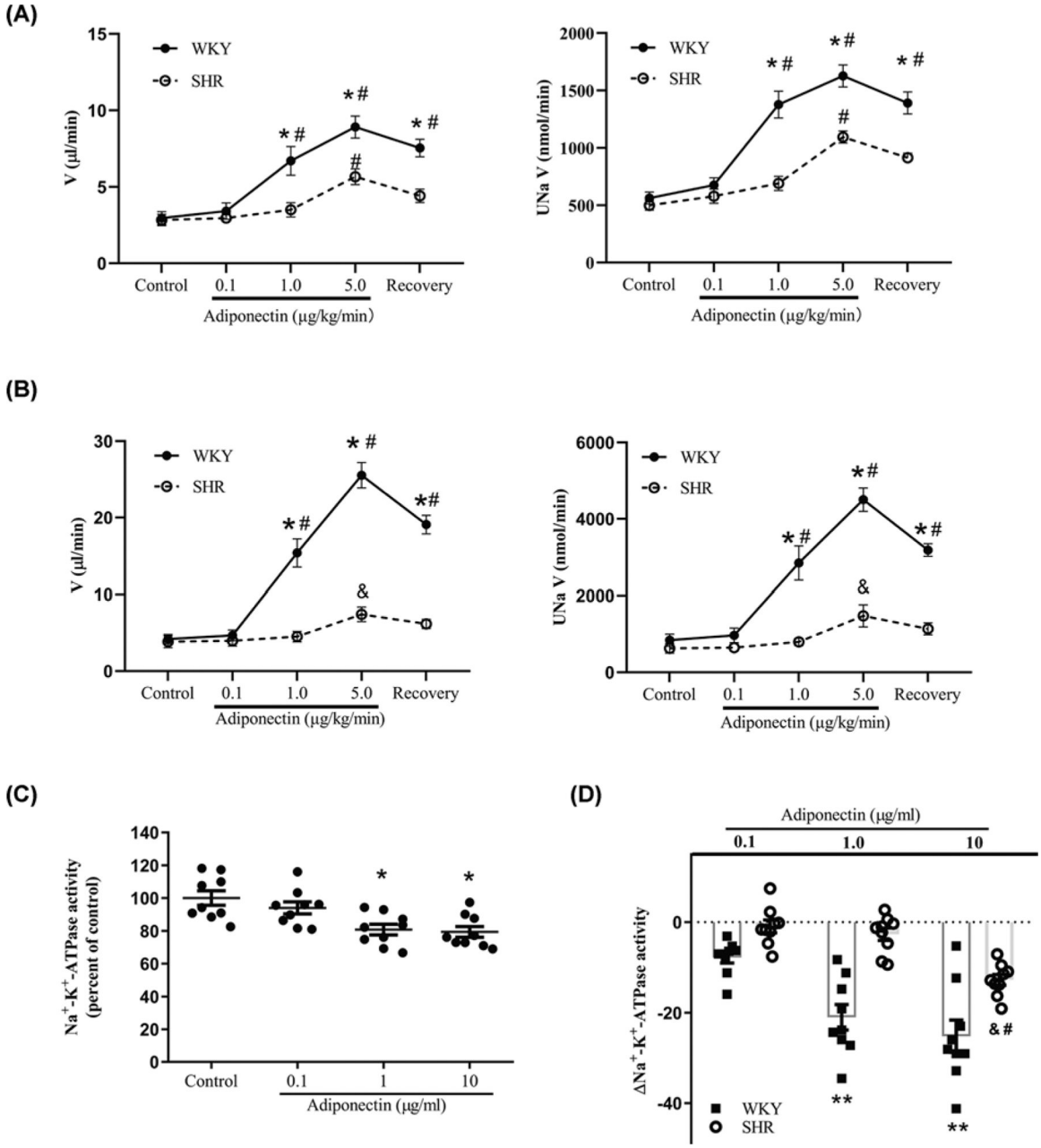

Figure 2. The impairment of adiponectin-mediated natriuresis and diuresis in SHRs

(**A,B**)The impairment of adiponectin-mediated natriuresis and diuresis in younger (A, 4-week-old) and older (B, 12-week-old) SHRs. Increasing doses of adiponectin (0.1–5.0 μg/kg per minute) were infused into the right renal artery, via the right suprarenal artery, in WKY and SHRs. Urine flow (V) and absolute sodium excretion (UNaV) were significantly increased in WKY rats but only slightly increased in SHRs at the dose of 5 μg/kg per minute (**P*<0.05 vs. WKY control, #*P*<0.05 vs. SHR, &*P*<0.05 vs. SHR control, *n*=6/group). (**C**) Adiponectin inhibits Na^+-K^+-ATPase activity in concentration-dependent manner in WKY RPT cells. WKY RPT cells were treated at the indicated concentrations (0.1–10 μg/ml, *n*=9) of adiponectin for 30 min. Results are expressed as percent change of control (**P*<0.05 vs.

control). (**D**) The inhibitory effect of adiponectin on Na^+-K^+-ATPase activity is reduced in SHR RPT cells. RPT cells from WKY and SHRs were treated with indicated concentrations of adiponectin; results are expressed as relative change of Na^+-K^+-ATPase activity from basal level (**P<0.01 vs. WKY control, $^{\#}P$<0.05 vs. others in SHR, $^{\&}$P<0.05 vs. the same concentration of adiponectin in WKY, n=9/group).

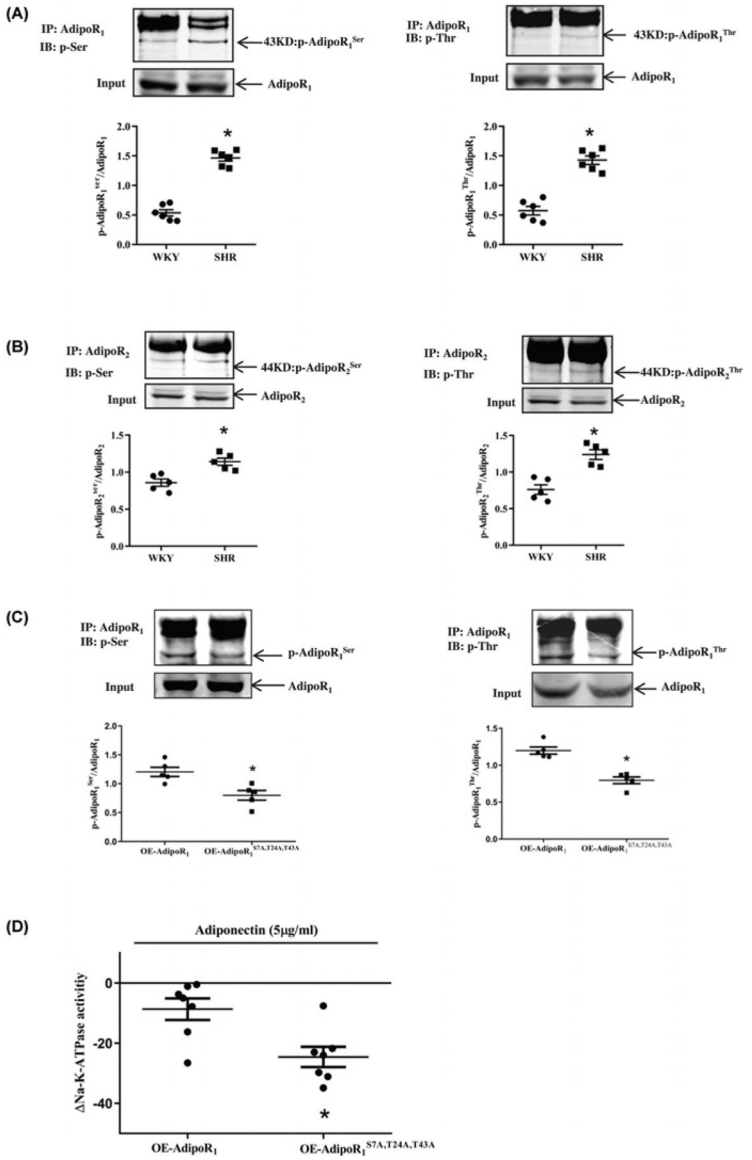

Figure 3. Effect of phosphorylation site mutation on AdipoR$_1$ phosphorylation and function in RPT cells from SHRs

(**A,B**) AdipoR$_1$ and AdipoR$_2$ phosphorylation in kidneys from 12-week-old WKY and SHRs. Renal cortex lysates were immunoprecipitated with anti-AdipoR$_1$ or anti-AdipoR$_2$ antibody and then immunoblotted with anti-phosphorylated threonine (p-Thr) or anti-phosphorylated serine (p-Ser) antibody. IB, immunoblotting; IP, immunoprecipitation (*P<0.05 vs. WKY, n=5–6/group). (**C**) Effect of phosphorylation site mutation on AdipoR$_1$ phosphorylation in SHR RPT cells. SHR RPT cells were transfected with wildtype or mutant (Ser^7 Thr^{24} Thr^{43} replaced by Ala) plasmids; AdipoR$_1$ phosphorylation was determined by immunoprecipitation. Cell lysates were immunoprecipitated with anti-AdipoR$_1$ antibody and then immunoblotted with anti-phosphorylated threonine (p-Thr) or anti-phosphorylated serine (p-Ser) antibody (*P<0.05 vs. wildtype plasmid transfection, n=5/group). (**D**) Effect of phosphorylation site mutation on adiponectin-mediated inhibition of Na^+-K^+-ATPase activity in SHR RPT cells. SHR RPT cells were transfected with WT

(OE-AdipoR$_1$) or mutant (OE-AdipoR$_1^{S7A, T24A, T43A}$; Ser^7 Thr^{24} Thr^{43} replaced by Ala) plasmids. Na^+-K^+-ATPase activity was determined in RPT cells treated with adiponectin (5 μg/ml) for 30 min (*P<0.05 vs. OE-AdipoR$_1$, n=9/group).

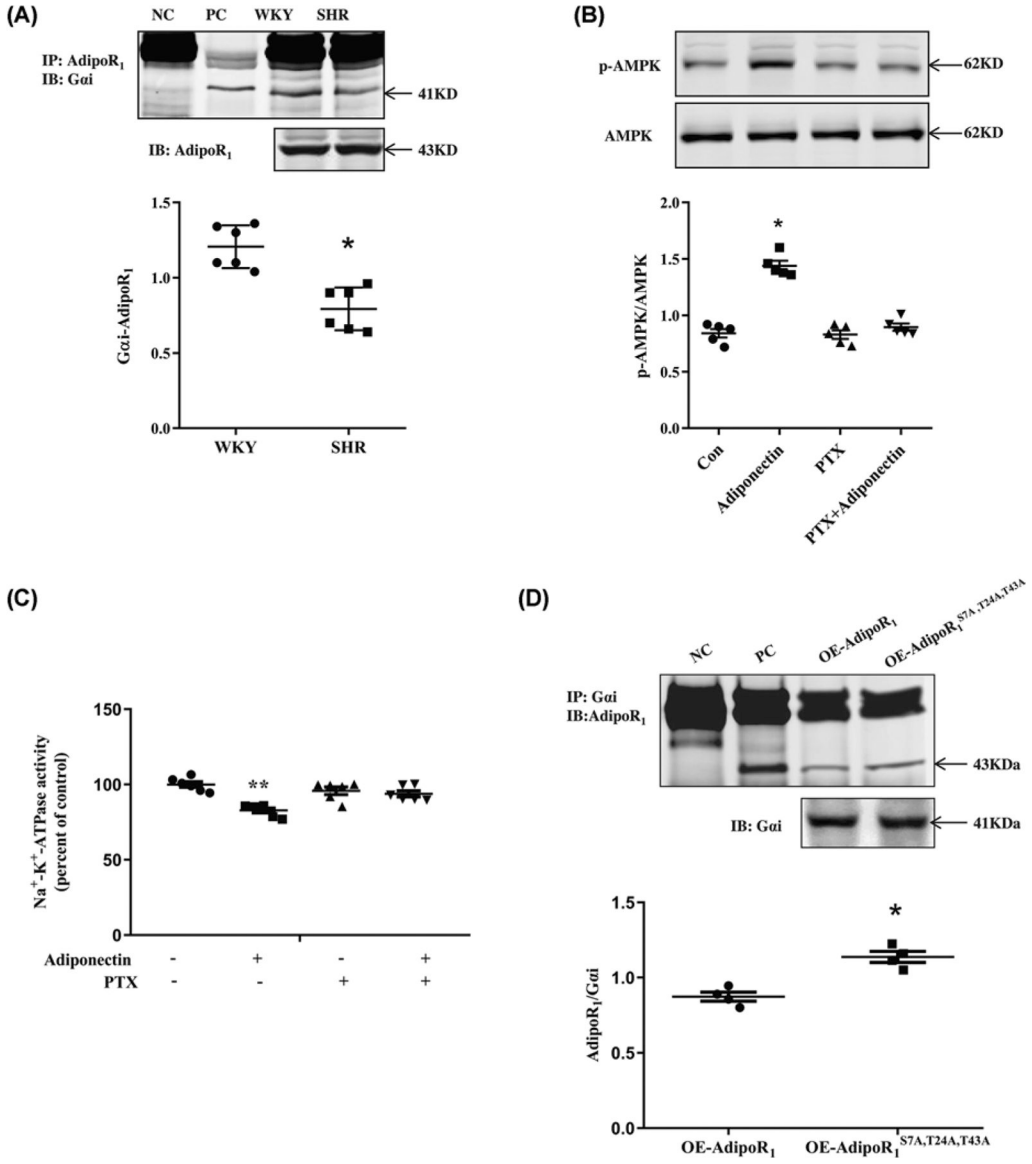

Figure 4. AdipoR$_1$ and Gαi uncoupling is a vital step leading to impaired natriuretic effect of adiponectin

(**A**) The coupling between AdipoR$_1$ and Gαi in kidneys from WKY and SHRs. Renal cortex homogenates were immunoprecipitated with anti-AdipoR$_1$ and then immunoblotted with anti-Gαi. For negative control, mouse IgG was used for immunoprecipitation. For positive control, anti-Gαi antibody was used for both immunoprecipitation and immunoblotting (*P<0.05 vs. WKY, n=6/group). (**B**) Adiponectin (Adipo)-induced up-regulation of AMPK phosphorylation (p-AMPK) was blocked by PTX, a Gαi inhibitor. RPT cells from WKY rats were pre-incubated with PTX (1 μg/ml) for 2 h and then treated with adiponectin (1 μg/ml, 30 min) (*P<0.05 vs. others, n=5/group). (**C**) Adiponectin-induced inhibitory effect on Na$^+$-K$^+$-ATPase activity in RPT cells was blocked by PTX, a Gαi inhibitor. RPT cells from WKY rats were pre-incubated with PTX (1 μg/ml) for 2 h and then treated with adiponectin (1 μg/ml, 30 min) (**P<0.01 vs. others, n=6/group). (**D**) Co-immunoprecipitation of AdipoR$_1$ and Gαi in SHR RPT cells transfected with WT or AdipoR$_1$ phosphorylation site

mutant plasmid. Cell lysates were immunoprecipitated with anti-Gαi antibody and then immunoblotted with anti-AdipoR$_1$. For negative control, mouse IgG was used for immunoprecipitation. For positive control, anti-Gαi antibody was used for both immunoprecipitation and immunoblotting (*P<0.05 vs. WT plasmid transfection, n=4/group).

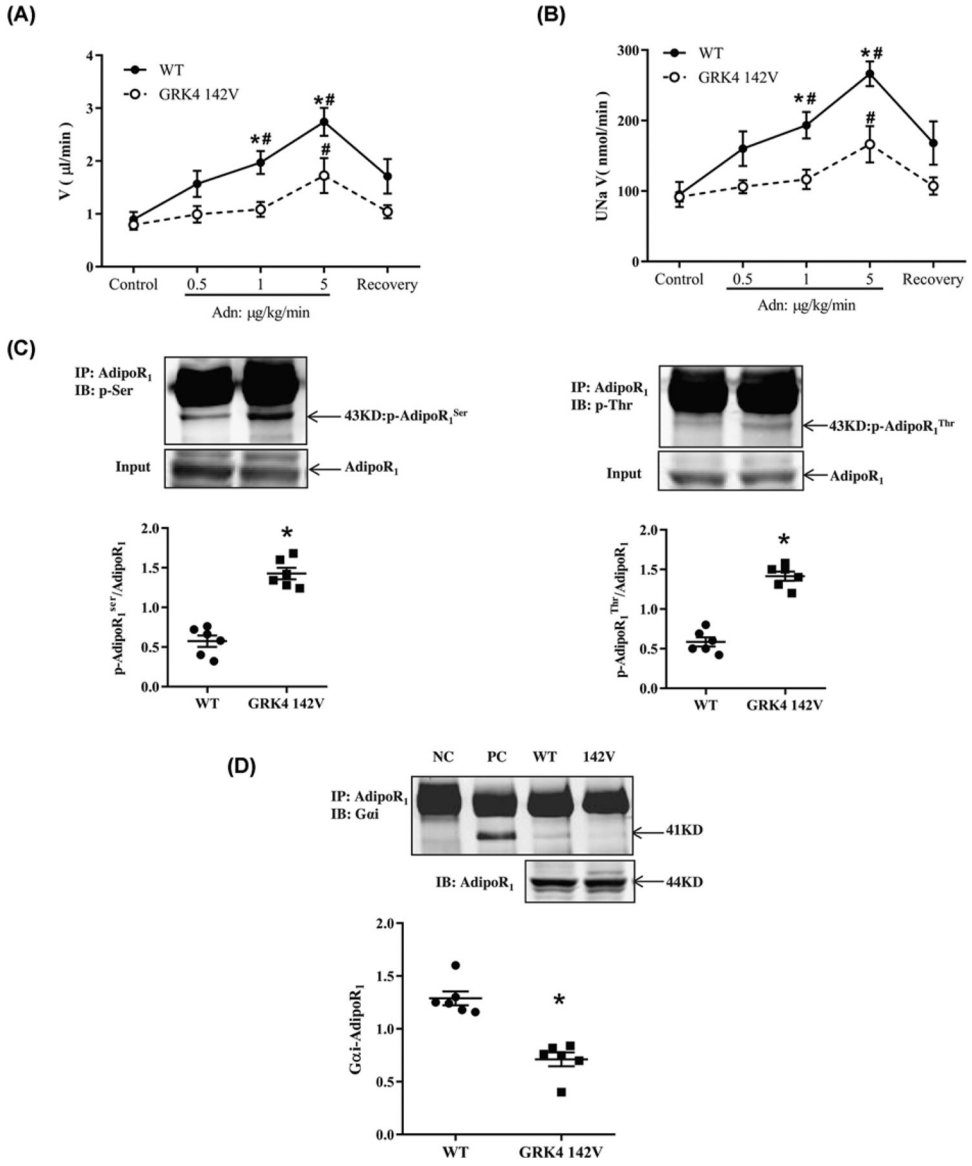

Figure 5. Effect of adiponectin on sodium excretion in GRK4γ142A>V transgenic and wildtype mice

(**A,B**) Effect of systemic infusion of adiponectin on sodium excretion in GRK4γ142A>V transgenic mice and GRK4γWT mice. Urine volume (A) and urinary sodium excretion (B) were monitored (*P<0.05 vs. GRK4γ142A>V, $^{\#}P$<0.05 vs. control, n=5–6). (**C**) Phosphorylation of $AdipoR_1$ at serine and threonine residues in kidneys from GRK4γ142A>V and GRK4γWT transgenic mice. The phosphorylation was determined by immunoprecipitation (*P<0.05 vs. GRK4γWT mice, n=6/group). (**D**) Linkage between $AdipoR_1$ and Gαi in kidneys from GRK4γ142A>V and GRK4γWT transgenic mice, determined by co-immunoprecipitation (*P<0.05 vs. GRK4γWT mice, n=6/group).

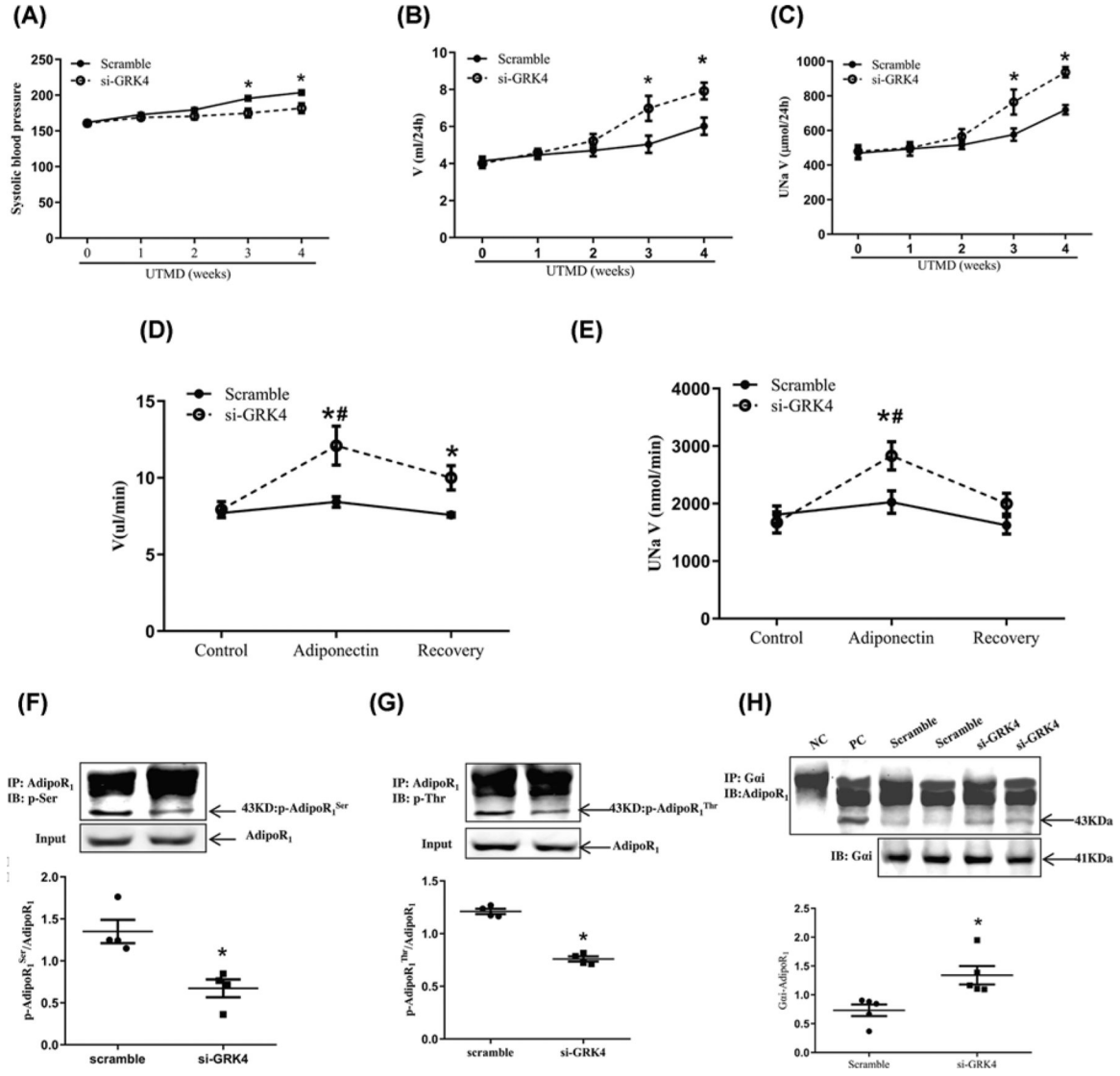

Figure 6. Suppression of renal GRK4 expression decreases blood pressure and recovers impaired adiponectin-induced sodium excretion in SHRs

(**A–C**) Effect of GRK4siRNA-UTMD on blood pressure and sodium excretion in SHRs. Eight-week-old SHRs were treated with UTMD (siRNA 1 OD per time, every 3 days for a total of ten treatments per rat). Blood pressure was measured by the tail-cuff method every week (A), 24-h urine was collected every week (B,C) (**P*<0.05 vs. scramble, *n*=5/group). (**D,E**) Effect of GRK4 siRNA-UTMD on adiponectin-mediated urine flow and sodium excretion in 12-week-old SHRs. Adiponectin (5.0 μg/kg per minute) was infused into the right renal artery, via the right suprarenal artery. Urine flow (V, D) and absolute sodium excretion (UNaV, E) were measured (**P*<0.05 vs. scramble, #*P*<0.05 vs. control, *n*=5/group). (**F,G**) Effect of GRK4 siRNA-UTMD on $AdipoR_1$ phosphorylation in kidneys from SHRs. $AdipoR_1$ phosphorylation at serine (F) and threonine (G) residues was determined by co-immunoprecipitation (**P*<0.05 vs. scramble, *n*=4/group). (**H**) The coupling between $AdipoR_1$ and Gαi after UTMD treatment in kidneys from SHRs. The kidney lysates were immunoprecipitated with anti-Gαi antibody and then immunoblotted with anti-$AdipoR_1$

antibody. For negative control, mouse IgG was used for immunoprecipitation. For positive control, anti-AdipoR$_1$ antibody was used for both immunoprecipitation and immunoblotting (*P<0.05 vs. scramble, n=5/group).