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Role of GRK4 in the regulation of the renal ETB receptor in hypertension

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Abstract

The endothelin receptor type B (ETBR) regulates water and electrolyte balance and blood pressure, in part, by inhibiting renal sodium transport. Our preliminary study found that the ETBR-mediated diuresis and natriuresis are impaired in hypertension with unknown mechanism. Persistently increased activity of G protein-coupled receptor kinase 4 (GRK4), caused by increased expression or genetic variants (eg, GRKγ142V), impairs the ability of the kidney to excrete a sodium load, in part, by impairing renal dopamine D_1 receptor function through persistent phosphorylation. Our present study found that although renal ETBR expression was not different between Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs), renal ETBR phosphorylation was higher in SHRs. The role of hyper-phosphorylation in impaired ETBR-function was supported by results in human (h) GRK4γ transgenic mice. Stimulation of ETBR by BQ3020-induced natriuresis in human (h) GRK4γ wild-type (WT) mice. However, in hGRK4γ 142V transgenic mice, the renal ETBR was hyperphosphorylated and ETBR-mediated natriuresis and diuresis were not evident. There were co-localization and co-immunoprecipitation of ETBR and GRK4 in renal proximal tubule (RPT) cells from both WKY and SHRs but was greater in the latter than the former group. SiRNA-mediated downregulation of GRK4 expression, recovered the

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AUTHOR CONTRIBUTIONS

Y. Liu and C. Lan conceived the project and designed the experiments; Y. Yang, M. Li, X. Zou, C. Chen, S. Zheng, C. Fu, K. Chen, and P.A. Jose conducted experiments; Y. Yang wrote the manuscript; P.A. Jose, C. Lan, and Y. Liu revised the manuscript. All authors reviewed and approved the manuscript.

DISCLOSURES

None.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

impaired inhibitory effect of ETBR on Na^+-K^+-ATPase activity in RPT cells from SHR. In vivo downregulation of renal GRK4 expression, via ultrasound-targeted microbubble destruction, decreased ETBR phosphorylation and restored ETBR-mediated natriuresis and diuresis in SHRs. This study provides a mechanism by which GRK4, via regulation of renal ETBR function, participates in the pathogenesis of hypertension.

Keywords

endothelin type B receptor; G protein-coupled receptor kinase 4; hypertension; natriuresis

1 | INTRODUCTION

Hypertension is a well-established risk factor for cardiovascular disease, affecting nearly 31% of the world's adults,[1] and leads to myocardial infarction, stroke, renal failure, and death, if not detected early and treated appropriately.[2] The kidney plays an important role in the long-term regulation of blood pressure by maintaining sodium homeostasis; hypertension may be caused by abnormal sodium chloride metabolism.[3,4] The increased sodium retention in hypertension is ascribed, at least in part, to enhanced ion transport in all segments of the nephron, which is regulated by numerous hormones and humoral factors, including angiotensin II, dopamine, and endothelin (ET)-1.[3,5,6]

ET-1 is a potent vasoactive peptide originally isolated from endothelial cells and acts by binding to the ET subtype A receptor (ETAR) and ET subtype B receptor (ETBR). In vascular smooth muscle cells, both ETAR and ETBR mediate the vasoconstrictor effects of ET-1, while ETBR, in endothelial cells, by releasing NO and prostacyclin, mediates the vasodilatory effect of ET-1.[7] The effect of exogenously administered ET-1 on sodium excretion is inconsistent.[6,8,9] This may be related to the opposing effects of ETAR and ETBR on sodium transport. In the kidney, ET-1 has diuretic and natriuretic effects that could be inhibited by an ETBR antagonist,[6,10] indicating that ETBR could be more important than ETAR in the regulation of renal sodium and water transport. Stimulation of ETBR in RPT cells suppresses the stimulatory effect of Na^+-K^+-ATPase on sodium transport, which may be one mechanism for the natriuretic effect of ETBR in WKY rats.[11–13] However, the inhibitory effect of ETBR on sodium transport is more evident in the collecting duct, where ETBR inhibits the activity of the epithelial sodium channel.[6] More and more studies have shown the importance of renal ET-1/ETBR axis in hypertension and alterations in renal ET-1 production may contribute to the hypertension in spontaneously hypertensive rats (SHRs).[14] ETBR deficiency in rats cause salt-sensitive hypertension that is due to increased activity of the epithelial sodium channel.[15] Indeed, the silencing of ETBR selectively in the renal collecting duct causes sodium retention and hypertension in mice.[16] Thus, the pharmacological stimulation of ETBR is a promising strategy for the therapy of hypertension. However, our previous study found an impaired response of RPT cells to ETBR agonist stimulation in SHRs,[17] but mechanisms of this impaired response are unknown. Therefore, determining the underlying mechanisms accounting for the renal ETBR dysfunction in the SHR have implications in the treatment of hypertension.

Similar to many hormone receptors, including angiotensin receptors and dopamine receptors, ETBR belongs to the family of G protein-coupled receptors (GPCRs). These receptor-regulated biological responses in many physiological situations can be regulated by G protein-coupled receptor kinases (GRKs). The GRK4-mediated regulation of the phosphorylation and function of the dopamine D_1 (D_1R) and D_3 (D_3R) receptors plays an important role in the pathogenesis of hypertension.[18,19] However, whether GRK4 plays a role in the dysfunction of ETBR in the SHR is unknown. In the present study, we found that in the SHR, the renal ETBR was hyper-phosphorylated with impaired diuretic and natriuretic responses to ETBR stimulation. Downregulation of GRK4 restored the ETBR-mediated diuresis and natriuresis in these SHRs, indicating that GRK4 could be targeted to recover the impaired GPCR function, including those of dopamine and ETBRs.

2 | METHODS

2.1 | Animals and surgical procedures

Adult (9–16 weeks old) male and female WKY and SHRs, weighing 280–300 g, were purchased from Shanghai SLRC Laboratory Animal Company (Shanghai, China). hGRK4γ WT and hGRK4γ 142V transgenic mice were generated as we previously reported.[20] Food, but not, water was withheld 24 hours before surgery. After the rats were anesthetized (intraperitoneal pentobarbital, 50 mg/kg), they were placed on a heating pad to maintain a constant body temperature of 37°C, and then, tracheotomized (PE-240). During the surgery, anesthesia was maintained by the infusion of pentobarbital (0.8 mg/100 g/hour). The femoral and external jugular veins were catheterized (PE-50) for fluid replacement while the left carotid artery was catheterized (PE-240) for monitoring the systemic arterial pressure. Then, the right and left ureters, exposed via an abdominal laparotomy, were catheterized (PE-10). The right suprarenal artery was catheterized (PE-10 heat-stretched to 180 μm) for vehicle/drug infusion (40 μL/min). Fluid losses during the surgical procedure (about 60 minutes) were replaced with 5% of albumin in normal saline at 1% body weight over 30 minutes. After a 120-minute equilibration period, urine was collected for five periods, at 40 minutes per period. An electrolyte analyzer (Histrong Medical Equipment, Shenzhen, Guangdong, China) was used to measure urinary sodium concentration. All animal work took place in Chongqing Institute of Cardiology (Chongqing, China) and was in accordance with protocols approved by the Medical Ethics Committee of the Third Military Medical University. All efforts were made to minimize animal suffering.

2.2 | Cell culture

Immortalized renal proximal tubule (RPT) cells from 4- to 8-week-old WKY and SHRs were cultured at 37°C in 95% of air/5% of CO_2, as we have previously reported.[21,22] Prior to the addition of drugs, the cells were incubated for 2 hours in Dulbecco's modified Eagle's medium/F-12 culture medium without fetal bovine serum (FBS), to make the cells quiescent.

2.3 | Small interfering RNA transfection in RPT cells

Small interfering RNA (siRNA) targeted against GRK4 mRNA (GRK4 siRNA) and its control, scrambled RNA, were synthesized and purified, as we have previously reported.[20] In brief, RPT cells growing in 6-well plates at about 60% confluence were transfected with

siRNA (50 nmol/L) using 6 μL of oligofectamine in Optimem medium (Thermo Fisher Scientific, Waltham, MA). After incubation for 24 hours, the Optimem medium was switched to growth medium and incubated for another 24 hours. Then, the cells were analyzed or used for additional experiments. Transfection of siRNA targeted other GRK family members was similar to GRK4 siRNA.

2.4 | Na^+-K^+-ATPase activity

Na^+-K^+-ATPase activity was the difference between ATP hydrolysis in the absence and presence of ouabain according, as we have previously described.[23] RPT cells were washed twice with 5 mL of ice-cold phosphate-free buffer and centrifuged at 3000 *g* for 10 minutes. Thereafter, the cells were lysed in 2 mL lysis buffer (5 mmol/L of $MgCl_2$, 2 mmol/L of $CaCl_2$, and 1 mmol/L of $NaHCO_3$) and centrifuged at 3000 *g* for 2 minutes. The supernatant was mixed with 1 mol/L NaI in an equal volume followed by centrifugation at 48 000 *g* for 25 minutes, and then, the supernatant was removed. The pellet (membrane fraction) was washed twice and re-suspended in 10 mM of Tris with 1 mM of EDTA (pH 7.4). The protein was quantified using the Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). 100 μL aliquots of the cell membrane fractions were transferred into 800 μL reaction mixture (75 mmol/L of NaCl, 5 mmol/L of KCl, 5 mmol/L of $MgCl_2$, 75 mmol/L of Tris-HCl, 1 mmol/L of Na_4EGTA, 30 mmol/L of histidine, 37.5 mmol/L of imidazole, and 6 mmol/L of sodium azide (pH 7.4), with or without 1 mmol/L of ouabain), followed by preincubation for 5 minutes in water bath at 37°C. The reaction was initiated by the addition of 100 μL Tris-ATP (endpoint concentration was 4 mmol/L) and terminated after 15 minutes of incubation at 37°C, by the addition of 50 μL of 50% trichloroacetic. To assess the ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures with ouabain. For quantification of the amount of phosphate produced, 1 mL of coloring reagent (10% of ammonium molybdate in 10 N of sulfuric acid + ferrous sulfate) was added to the reaction mixture, and then, mixed thoroughly followed by centrifugation at 3000 *g* for 10 minutes. Formation of phosphomolyb-date was quantified spectrophotometrically at 740 nm with a standard curve prepared from K_2HPO_4. Na^+ - K -ATPase activity was measured as the difference between total and ouabain-insensitive ATPase activity and expressed as μmol phosphate released per mg protein per hour.

2.5 | Ultrasound-targeted microbubble destruction

Small interfering RNA (siRNA) targeted against rat GRK4 mRNA (GRK4 siRNA) and its control, scrambled RNA, were synthesized and purified, as previously reported.[20] Poly-L-lysine (PLL) and the electrostatic adsorption method were used to prepare microbubbles carrying GRK4 siRNA, as previously described.[24,25] In brief, a PLL solution (1 mg/mL) was prepared with sterile double-distilled water and mixed with equal volume of blank microbubbles followed by incubation at 4°C for 30 minutes. Phosphate-buffered saline (PBS) was used to wash the mixture twice to remove any unbound PLL. Thereafter, the microbubbles solution was mixed with GRK4 siRNA and incubated at 4°C for 30 minutes. Then, 200 mL of PBS were added to the mixture and unbound GRK4 siRNA was removed from the suspension after washing by centrifugation, 600 rpm for 3 minutes × 2.

Ultrasound-targeted microbubble destruction (UTMD) of GRK4 in the kidney of SHRs was performed following the method we have previously reported.[25] In brief, the rats were anesthetized by the intraperitoneal injection of 2% of pentobarbital sodium at a dose of 50 mg/kg and placed on a heating pad to maintain a constant body temperature of 37°C. The kidneys were localized and irradiated for 5 minutes, using the 9L4 linear array probe of an ultrasound imaging system. The microbubbles carrying GRK4 siRNA were shaken in a custom-built oscillation apparatus for 45 seconds to form a microbubble suspension and injected within 30 seconds into the tail vein through a 26-gauge needle connected to a 1-mL syringe. The UTMD treatments were performed every 3 days for a total of six treatments. After the UTMD treatment, the rats were euthanized using an overdose of isoflurane (5%). The kidneys were harvested to examine the mRNA and protein expression of GRK4 and ETBR.

Other rats were used to investigate ETBR-mediated diuresis and natriuresis. In brief, the right suprarenal arteries of the pentobarbital-anesthetized rats were infused with increasing doses of the BQ3020 (0.1, 0.5, and 1.0 μg/kg/min × 40 minutes), which is an endothelin agonist selective for ETBR,[26] at a rate of 0.04 mL/min, preceded and followed by a recovery period of 40 minutes, in which only vehicle (normal saline) was infused. The vehicle, infused at the same rate in other rats, served as control. The urine was collected for five periods, 40 minutes per period.

2.6 | Reverse transcription polymerase chain reaction

Total RNA was extracted from the RPT cells and kidney samples using RNAiso Plus (TAKARA, Tokyo, Japan), following the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed with an RT-PCR kit (TOYOBO, Osaka, Japan). Quantitative real-time PCR of GRK4 and GAPDH, which served as the house-keeping gene control, was carried out using a Bio-Rad CFX96 Touch Real-Time PCR Detection System. The melting curves and quantification were analyzed using Bio-RAD software. A comparative cycle threshold method was used to determine the relative quantity of RNA expression. The PCR of each sample was repeated at least three times.

2.7 | Immunoprecipitation

The renal cortex or RPT cells were lysed, and then, equal amounts of lysates (300 μg protein/mL supernatant) were incubated with affinity-purified mouse anti-GRK4 antibody (5 μg/mL, GRK4/ETBR coimmunoprecipitation; Santa Cruz Biotechnology, Dallas, TX) or mouse anti-phosphoserine or mouse ETBR antibody (5 μg/mL, ETBR phosphorylation; Abcam, Cambridge, UK) for 1 hour and protein G plus-agarose overnight at 4°C. The immunoprecipitates were subjected to immunoblotting with rabbit anti-ETBR antibody (1:1000; Abcam) or anti-phosphoserine antibody. Anti-ETBR antibody or anti-phosphoserine antibody (positive control) and IgG (negative control) were used as the immunoprecipitants to evaluate the specificity of the bands found on the immunoblots.

2.8 | Immunoblotting

Cell or tissue lysates were prepared using RIPA buffer (150 mM of NaCl, 50 mM of Tris, 0.1% of SDS, 1% of Nonidet P-40, 0.5% of sodium deoxycholate, and protease inhibitor

mixture; Roche). Protein concentrations were quantified with the Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Uniform amounts of protein were subjected to SDS-PAGE with 8% of polyacrylamide gel, and then, electrotransferred onto polyvinylidene difluoride (PVDF) membranes followed by blocking for 1 hour with TBS (Tris-buffered saline), containing 5% of nonfat dry milk. The blotted membranes were incubated overnight at 4°C with the following primary antibodies: anti-GAPDH antibody (1:1000; Abcam), anti-GRK4 antibody (1:800; Santa Cruz Biotechnology), and anti-ETBR antibody (1:1000; Abcam). The membranes were washed three times with TBS and incubated with corresponding diluted (1: 15 000) secondary antibody (donkey anti-rabbit IR Dye 800, donkey anti-mouse IR Dye 800; Li-Cor Biosciences, Lincoln, NE) for 1 hour at room temperature. After washing, the bands were visualized by the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). The relative intensities of the protein bands were analyzed by densitometry using the Quantity One image analysis software. GAPDH was used as an internal control to normalize the densitometric intensity corresponding to each band. All primary antibodies were validated by western blot by the manufacturers and as stated in the immunoprecipitation protocol. All experiments were repeated at least three times.

2.9 | Confocal microscopy

The RPT cells, grown on coverslips, were fixed with 4% of paraformaldehyde (10 minutes) and permeabilized with 0.3% of Triton X-100 (30 minutes). The cells were double-immunostained for ETBR and GRK4, using rabbit anti-ETBR antibody (1:100) and mouse anti-GRK4 antibody (1:100), respectively, and then, with Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) secondary antibody (1:200; Thermo Fisher Scientific) and Alexa Fluor 546-conjugated goat anti-rabbit IgG (H + L) secondary antibody (1:200; Thermo Fisher Scientific), respectively. Images were obtained using laser confocal microscopy and evaluated using the Olympus Fluoview FV300 version 3C Acquisition Software.

2.10 | Statistical analysis

The data are expressed as mean ± SEM. Comparison within groups was made using repeated measures analysis of variance ANOVA with Holm-Sidak test (or paired *t* test when only two groups were compared). Comparison among groups was made using one-way factorial ANOVA with Holm-Sidak test (or *t* test when only two groups were compared). A value of $P < .05$ was considered as statistically significant.

3 | RESULTS

3.1 | Diuretic and natriuretic effects of ETBR are impaired in SHRs

Our previous study found that stimulation of ETBR inhibited Na^+-K^+ ATPase activity in RPT cells from WKY rats.[11] The present study further showed that the infusion of the ETBR agonist BQ3020, via the right suprarenal artery, induced a dose-dependent (0.1, 0.5, and 1.0 μg/kg/min) diuresis and natriuresis in male WKY rats that persisted for forty minutes after stopping the drug infusion (Figures 1A,B). By contrast, in male SHRs, the BQ3020-mediated diuresis and natriuresis were not observed (Figures 1A,B). The intrarenal infusion of BQ3020 did not affect systemic blood pressure in WKY and SHRs (data not

shown). Consistent with the results from male rats, BQ3020-mediated diuresis and natriuresis were also observed in female WKY rats, but not in female SHRs (Figures S1A,B), indicating ETBR-mediated diuresis and natriuresis are impaired in both male and female SHR rats.

ETBR, being a G protein-coupled receptor (GPCR), is regulated by phosphorylation, one posttranslational modification that regulates GPCR function.[27] Our present study found that although there was no difference in ETBR expression in homogenates from renal cortices of WKY and SHRs, renal cortical ETBR phosphorylation was greater in SHRs than WKY rats (Figures 2A,B, and S2), indicating that renal cortical ETBR hyper-phosphorylation could be the underlying mechanism for the impaired renal ETBR function in SHRs.

3.2 | ETBR-mediated diuresis and natriuresis are impaired in GRK4 transgenic mice

The phosphorylation of GPCRs is regulated by GRKs, specifically GRK4, as we have reported for D_1R, D_3R, and AT_1R.[18,19,28] Consistent with previous reports, our present study found that the mRNA and protein expressions of GRK4 were higher in the renal cortex of SHRs than that in WKY rats (Figures 2C,D). To further determine whether or not GRK4 is involved in the regulation of ETBR function, we used GRK4 variant, hGRK4γ 142V transgenic mice. Consistent with previous reports,[18,20,28] hGRK4γ 142V transgenic mice had higher blood pressure than hGRK4γ WT transgenic mice (Figure 3A) fed normal salt diet. However, both urine flow (V) and absolute sodium excretion (UNaV), in the basal state, were not different between hGRK4γ 142V and hGRK4γ WT transgenic mice (Figures 3B,C), which is consistent with a previous report.[28] The intravenous infusion of the ETBR agonist BQ3020 (0.1, 0.5, and 1.0 μg/kg/minute/40 minutes) did not affect systemic blood pressure in hGRK4γ 142V and hGRK4γ WT transgenic mice (data not shown). However, BQ3020 infusion induced a diuresis and natriuresis in hGRK4γ WT transgenic mice, but not in hGRK4γ 142V transgenic mice (Figures 3D,E), indicating the important role of GRK4 in the regulation of ETBR function. In addition, the impaired natriuresis and diuresis in hGRK4γ 142V transgenic mice was accompanied by increased phosphorylation of renal ETBR (Figures 3F and S2), further supporting a role of GRK4 in regulating the ETBR.

3.3 | GRK4 directly interacts with ETBR in RPT cells from WKY and SHRs

The interaction between ETBR and GRK4 was investigated by laser confocal immunofluorescence microscopy and coimmunoprecipitation studies. There was co-localization of ETBR and GRK4 in RPT cells from both WKY and SHRs (Figure 4A). A direct interaction between GRK4 and ETBR was confirmed by co-immunoprecipitation study (Figure 4B). Notably, the results showed there was more ETBR linked with GRK4 in SHR than WKY RPT cells, indicated by more co-localization and coimmunoprecipitation in SHR than WKY RPT cells (Figures 4A,B). This increased interaction between ETBR and GRK4 could be a factor in the increased phosphorylation of renal ETBR in SHRs.

3.4 | Downregulation of GRK4 expression restores ETBR-mediated inhibition of Na^+-K^+-ATPase activity in RPT cells from SHRs

The preceding data show that GRK4 is a major reason for the ETBR dysfunction in SHRs. We, therefore, tested the hypothesis that the renal ETBR function in the SHR could be

restored by the downregulation of renal GRK4 expression. Our present study found that stimulation of ETBR by BQ3020 (8 mmol/L) inhibited Na^+-K^+ ATPase in WKY RPT cells, which is consistent with our previous report.[11] However, in SHR RPT cells, the inhibitory effect of ETBR on Na^+-K^+ ATPase activity was not observed (Figure 5A). Note also that the basal RPT cell Na^+-K^+ ATPase activity is higher in the SHR than WKY rat. After downregulation of GRK4 with GRK4 siRNA (Figure 5B), the impaired inhibitory effect of the ETBR agonist BQ2020 on Na^+-K^+ ATPase activity in SHR RPT cells was restored to some degree (Figure 5C). This in vitro study was corroborated by an in vivo study. The UTMD-targeted silencing of renal GRK4, using GRK4 siRNA, decreased renal GRK4 expression (Figure 5D). Consistent with in vitro results, the downregulation of renal GRK4 expression restored the ETBR-mediated diuresis and natriuresis in SHRs that persisted after stopping the ETBR agonist infusion (Figures 5E,F). In addition, the downregulation of renal GRK4 also lowered the phosphorylation level of renal ETBR in SHRs (Figures 5G and S3). These data indicate that by improving ETBR-mediated diuresis and natriuresis in the SHR, GRK4 may be a promising therapeutic target in hypertension. We also explored whether other GRKs are also involved in regulation of ETBR phosphorylation by downregulation of GRK2–6 with siRNA in SHR RPT cells (Figure S4A). We found downregulation of GRK4 significantly lowered the phosphorylation level of ETBR, downregulation of GRK2 slighted lowered the phosphorylation level of ETBR but downregulation of other GRKs didn't have effect on phosphorylation of ETBR (Figure S4B), indicating GRK4 is the major GRK family number accounting for regulation of renal ETBR.

4 | DISCUSSION

ETBR has been found to play an important role in the regulation of blood pressure.[6–10,29,30] Stimulation of vascular ETBR, which is mainly located in the endothelium, causes vasorelaxation by enhancing nitric oxide production.[7,29] Relative to other organs, the highest concentration of ETBR is found in the kidney and it is essential in the long-term regulation of blood pressure by directly inhibiting renal sodium transport.[6–12,15,16,29,30]. Genetic disruption or pharmacological inhibition of the ETBR causes salt-sensitive hypertension in rats and mice.[15,16] This is especially true with the collecting duct-selective deletion of ETBR in mice.[16] We have reported that stimulation of ETBR decreased Na^+-K^+-ATPase activity in RPT cells from the WKY but not SHR,[17] suggesting a dysfunction of renal ETBR in hypertension. In our current study, we found that renal ETBR-mediated diuresis and natriuresis are impaired in SHRs, indicating a role of ETBR dysfunction in the pathogenesis of hypertension.

As aforementioned, ETBR belongs to the family of GPCRs; their phosphorylation is a common mechanism that desensitizes their function.[25,27,31] Our present study found that ETBR phosphorylation is higher in kidneys from SHRs than WKY rats, which may explain the dysfunction of renal ETBR in hypertension.[17] However, this supposition needs to be confirmed, that is, decreasing the hyper-phosphorylation of ETBR, per se, should improve ETBR function and ameliorate the hypertension. The exact phosphorylation site of ETBR and the effect of its hyperphosphorylation, per se, on renal sodium handling, also need to be investigated.

GRK4, one of the members GRK family, is expressed in a limited number of tissues, for example, artery, bone, cerebellum, heart, kidney, myometrium, small intestines, and testes, unlike GRK2, GRK3, GRK5, and GRK6, which are ubiquitously expressed.[32] The GRK4 locus (4p16.3) is linked to and GRK4 gene variants are associated with essential hypertension, in several ethnic groups.[33–35] Our previous study found that renal D_1R and D_3R functions are impaired in hypertension,[18,19,28,32,36,37] which is ascribed, in part, to the their persistent hyper-phosphorylation caused by increased expression of GRK4 in the kidney in rats[25,36] and GRK4 gene variants, such as GRK4γ142V in humans.[28] The persistent phosphorylation of GPCRs leads to their un-coupling from their G protein/effector complex and impairment of their function, D_1R and D_3R, for example.[25,28,36–39] In the present study, we found that renal GRK4 expression was, indeed, increased in SHRs, which was consistent with previous studies.[25,36] Therefore, we speculated that increased GRK4 expression and/or activity may have contributed to the increased renal ETBR phosphorylation in the SHR and hGRK4γ142V transgenic mice. Indeed, our present study showed that renal ETBR is hyperphosphorylated in the basal state and ETBR-mediated diuresis and natriuresis were impaired in hGRK4γ142V transgenic mice. Moreover, the downregulation of GRK4 expression in kidney of SHRs, via UTMD, decreased the basal renal ETBR phosphorylation and restored ETBR-mediated diuresis and natriuresis. We also found that the impaired inhibitory effect of ETBR on Na^+-K^+-ATPase activity in RPT cells from SHRs was restored by decreasing GRK4 expression, using GRK4 siRNA.

In summary, the present study showed that the diuretic and natriuretic effects of renal ETBR are impaired in the hypertensive state and caused by GRK4-mediated hyper-phosphorylation of ETBR. Renal downregulation of GRK4 partially restored the impaired diuretic and natriuretic effects of ETBR in SHRs, highlighting GRK4 as a promising target for restoring GPCR function, including ETBR, in the treatment of hypertension.

4.1 | Clinical Perspectives

1. Our preliminary study found that the ETBR-mediated diuresis and natriuresis is impaired in hypertension. However, the underlying mechanism causing this impairment is not clear.
2. We found impaired diuretic and natriuretic effects of renal ETBR in the hypertensive state were caused by GRK4-mediated hyper-phosphorylation of ETBR and renal downregulation of GRK4 partially restored the impaired diuretic and natriuretic effects of ETBR in SHRs
3. Our data provide strong evidence and suggest GRK4 as a promising target for restoring GPCR function, including ETBR, in the treatment of hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

D1R	dopamine D1 receptor
D3R	dopamine D3 receptor
ET	endothelin
ETAR	ET subtype A receptor
ETBR	endothelin receptor type B
FBS	fetal bovine serum
GPCRs	G protein-coupled receptors
GRK4	G protein-coupled receptor kinase 4
GRKs	G protein-coupled receptor kinases
PBS	phosphate-buffered saline
PLL	poly-L-lysine
PVDF	polyvinylidene difluoride
RPT	renal proximal tubule
RT-PCR	reverse transcription polymerase chain reaction
SHRs	spontaneously hypertensive rats
siRNA	small interfering RNA
TBS	tris-buffered saline
UNaV	absolute sodium excretion
UTMD	ultrasound-targeted microbubble destruction
V	urine flow
WKY	Wistar-Kyoto
WT	wild-type

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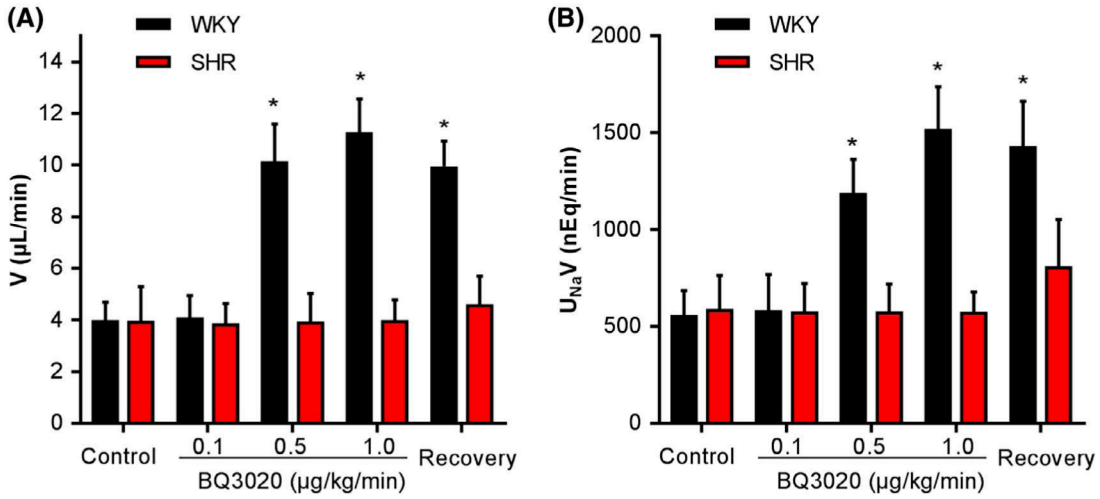

FIGURE 1.

Effect of the intrarenal arterial infusion of the ETBR agonist BQ3020 on urine flow and sodium excretion in male WKY and SHRs. A, Urine flow (V). B, Absolute sodium excretion ($U_{Na}V$). Varying doses (0.1, 0.5, and 1.0 µg/kg/min) of the ETBR agonist, BQ-3020, were infused at a rate of 0.04 mL/min for 40 minutes, preceded by a control period (40 minutes), and followed by a recovery period (40 minutes), in which the vehicle, normal saline, alone, was infused into the right supra-renal artery of anesthetized WKY and SHRs. *$P < .05$ vs control (N = 6/group). Data are expressed as Mean ± SE

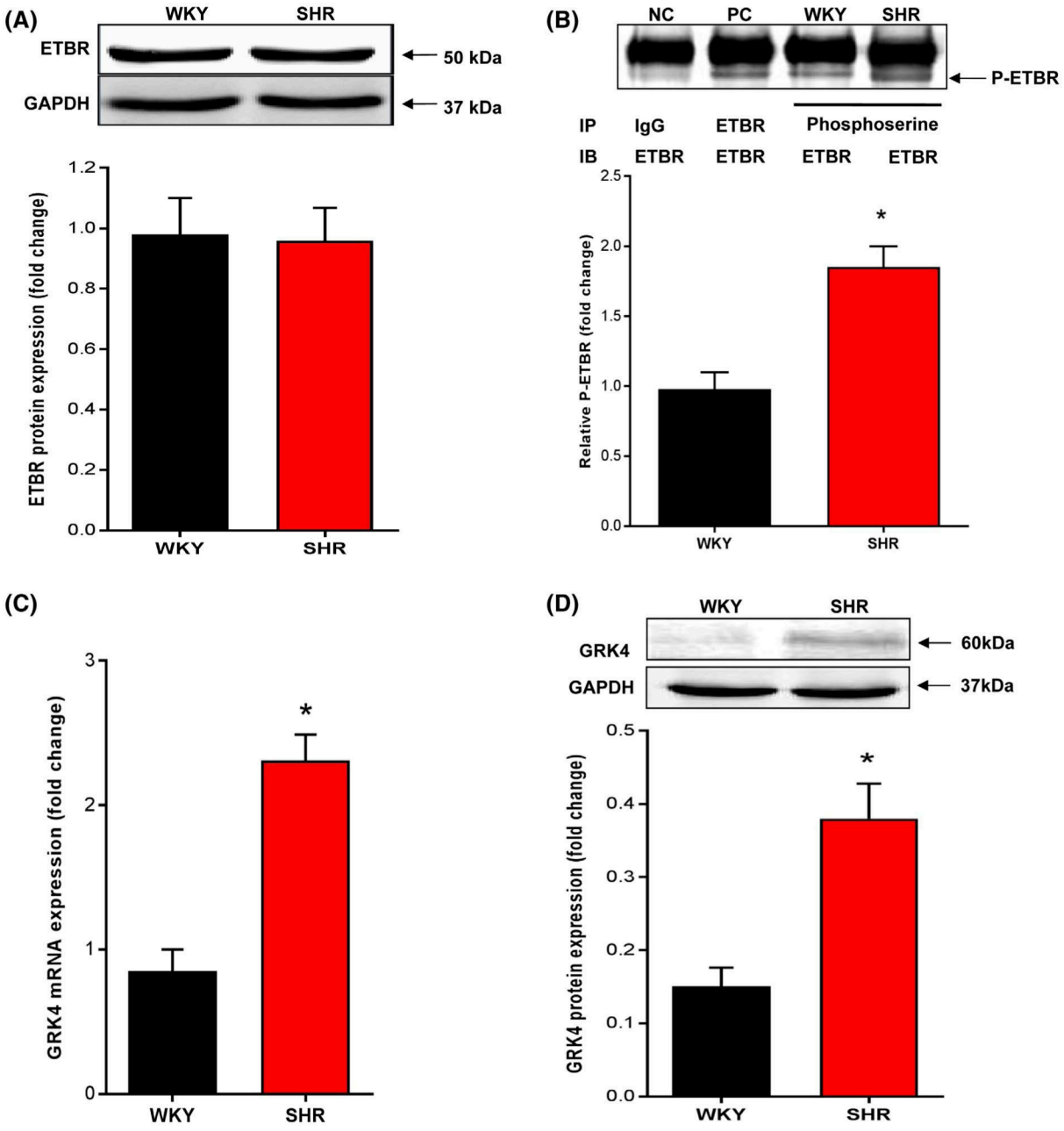

FIGURE 2.

ETBR phosphorylation and GRK4 expression in renal cortex of WKY and SHRs. A, ETBR protein expression determined by immunoblotting. Results are expressed as the ratio of ETBR and GAPDH densities (N = 5/group, P = NS). B, Renal cortical ETBR phosphorylation determined by co-immunoprecipitation with immunoblotting. Renal cortical lysates were prepared (renal cortex of WKY rats for negative and positive control group), and mouse anti-phosphoserine antibody was used for immunoprecipitation, with normal mouse IgG as negative control, and mouse anti-ETBR antibody as positive control. The immune complexes were precipitated using agarose-A/G beads and immunoblotted for ETBR. NC indicates negative control and PC indicates positive control. *$P < .05$ vs WKY

(N = 5/group). C and D, GRK4 mRNA and protein expressions in renal cortex of WKY and SHRs. *$P < .05$ vs WKY (N = 5/group). Data are expressed as Mean ± SE

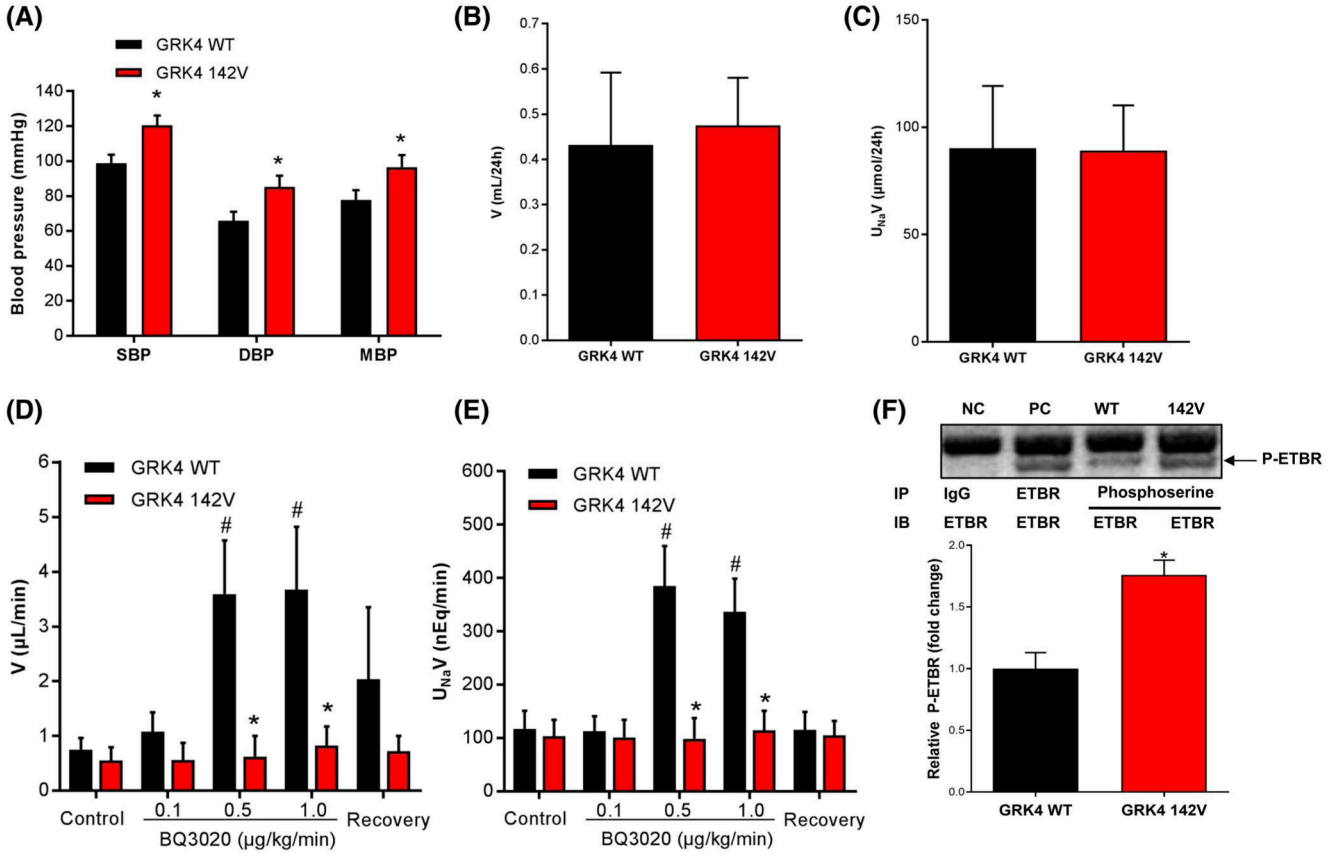

FIGURE 3.

Effect of the intrarenal arterial infusion of the ETBR agonist BQ3020 on urine flow and sodium excretion in hGRK4γ WT and hGRK4γ 142V transgenic mice. A, Systolic, diastolic, and mean arterial blood pressures (SBP, DBP, and MBP, respectively), measured under pentobarbital anesthesia in hGRK4γ WT and hGRK4γ 142V transgenic mice, $*P < .05$ vs WT (N = 5/group). B and C, Basal urine flow (24 hours, V) and basal absolute sodium excretion (24 hours, $U_{Na}V$). P = NS (N = 5/group). D and E, Urine flow (V) and absolute sodium excretion ($U_{Na}V$). Varying doses (0.1, 0.5, 1.0 μg/kg/min) of ETBR agonist, BQ-3020 were infused at a rate of 0.04 mL/min for 40 minutes, preceded by a control period (40 minutes), and followed by a recovery period (40 minutes) in which the vehicle, normal saline, alone, was infused via the right supra-renal artery of anesthetized mice. $^{\#}P < .05$ vs control. $*P < .05$ vs WT (N = 5/group). F, Renal cortical ETBR phosphorylation determined by coimmunoprecipitation with immunoblotting. Renal cortical lysates were prepared (renal cortex of hGRK4γ 142V transgenic mice for negative and positive control group), and mouse anti-phosphoserine antibody was used for immunoprecipitation, with normal mouse IgG as negative control, and mouse anti-ETBR antibody as positive control. The immune complexes were precipitated using agarose-A/G beads and immunoblotted for ETBR. NC indicates negative control and PC indicates positive control. $*P < .05$ vs WT (N = 4/group). Data are expressed as Mean ± SE

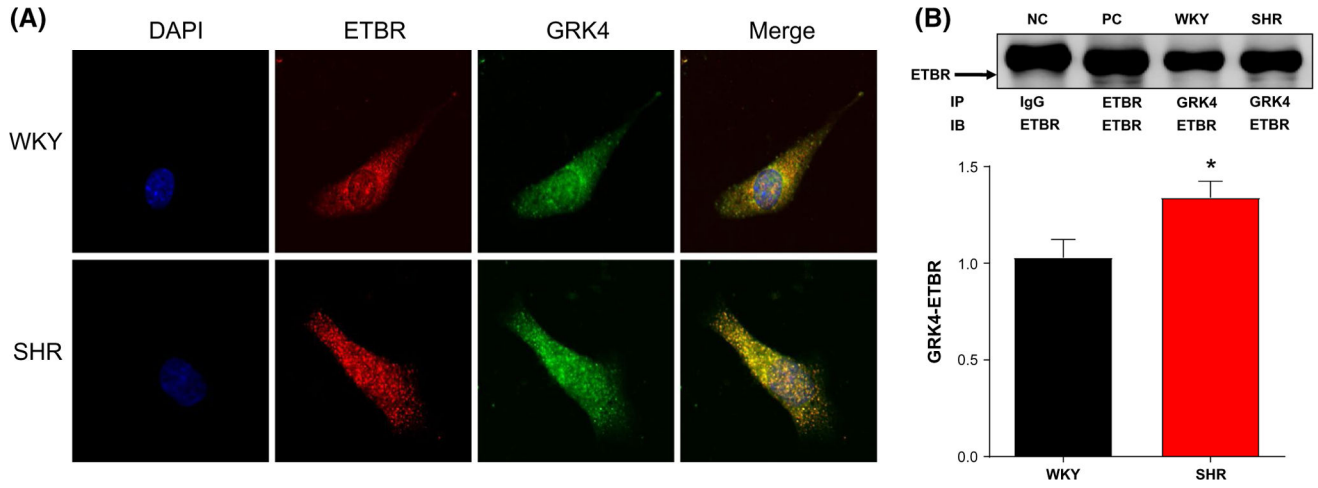

FIGURE 4.

Interaction between ETBR and GRK4 in WKY and SHR RPT cells. A, Colocalization of ETBR and GRK4 in RPT cells from WKY and SHRs. Colocalization appears as yellow after merging the images of Alexa Fluor 546-tagged ETBR (red) and Alexa Fluor 488-tagged GRK4 (green). B, Co-immunoprecipitation of GRK4 and ETBR in RPT cells from WKY and SHRs. The cell lysates (lysates of RPT cells from SHR for negative and positive control group) were immunoprecipitated with mouse anti-GRK4 antibody, with normal mouse IgG as negative control and mouse anti-ETBR antibody as positive control, and then, immunoblotted with ETBR antibody. NC indicates negative control and PC indicates positive control. $*P < .05$ vs WKY (N = 4/group). Data are expressed as Mean ± SE

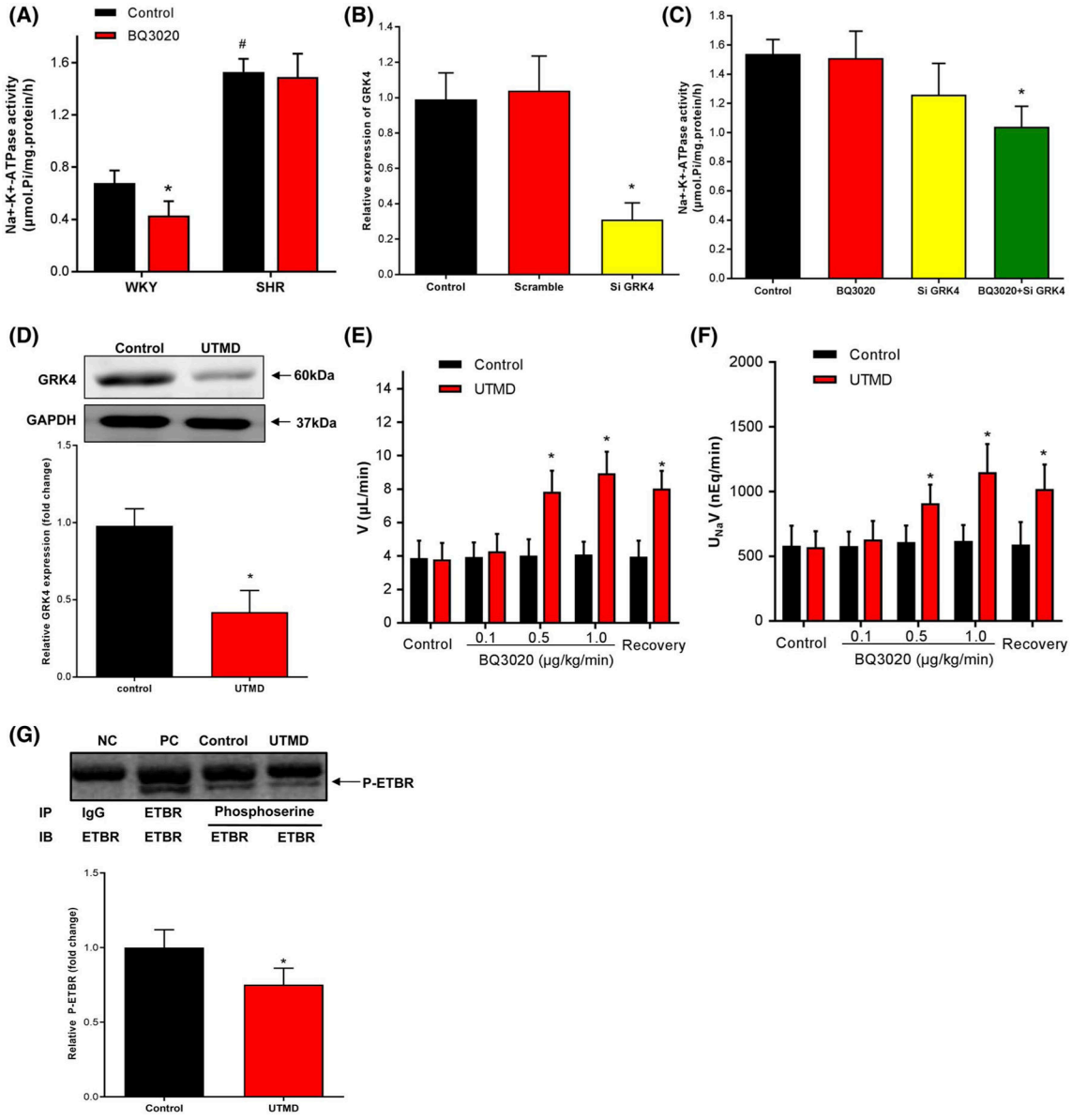

FIGURE 5.

Effect of GRK4 silencing on ETBR function. A, Effect of ETBR BQ3020 on Na^+-K^+-ATPase activity in RPT cells from WKY and SHRs. The cells were incubated with BQ3020 (10^{-8} M) or vehicle (dH_2O) for 30 minutes. Results are expressed as μmol phosphate released per mg protein per hour. $^{\#}P < .05$ vs WKY. $^{*}P < .05$ vs control (N = 6/group). B, Effect of GRK4-specific siRNA on GRK4 expression in RPT cells from SHRs. GRK4-specific siRNA (5 nM) or nonsilencing "mock" siRNA (negative control) was transfected into RPT cells for 72 hours. Untransfected cells served as an additional negative control. The mRNA expression of GRK4 related to control (RPT cells) was quantified using quantitative real-time PCR. $^{*}P < .05$ vs others (N = 4/group). C, Effect of the ETBR agonist BQ3020 on Na^+-K^+-ATPase activity in RPT cells from SHRs with or without GRK4 silencing with siRNA. The cells were incubated with BQ3020 (10^{-8} M) or vehicle (dH_2O) for 30 minutes.

Results are expressed as μmol phosphate released per mg protein per hour. *$P < .05$ vs control (N = 6/group). D, Effect of renal UTMD-mediated GRK4 siRNA delivery on renal cortical GRK4 protein expression in SHRs. *P < 0.05 vs control (N = 4/group). E and F: Urine flow (V) and absolute sodium excretion ($U_{Na}V$) in SHRs after downregulation of renal GRK4 expression by UTMD. The SHRs were subjected to UTMD-targeted GRK4 siRNA delivery to the kidney, given every 3 days for a total of six treatments. After the UTMD treatment, varying doses (0.1, 0.5, 1.0 μg/kg/min) of the ETBR agonist BQ-3020 were infused at a rate of 0.04 mL/min for 40 minutes, preceded by a control period (40 minutes), and followed by a recovery period (40 minutes) in which the vehicle, normal saline, alone was infused via the right supra-renal artery of anesthetized mice. *$P < .05$ vs control (N = 6/group). G, Renal cortical ETBR phosphorylation determined by co-immunoprecipitation with immunoblotting. Renal cortical lysates were prepared (renal cortex of SHR rats for negative and positive control group), and mouse anti-phosphoserine antibody was used for immunoprecipitation, with normal mouse IgG as negative control, and mouse anti-ETBR antibody as positive control. The immune complexes were precipitated using agarose-A/G beads and immunoblotted for ETBR. NC indicates negative control and PC indicates positive control.*$P < .05$ vs control (N = 4/group). Data are expressed as Mean ± SE