Role of GRK4 in the Regulation of Arterial \( AT_1 \) Receptor in Hypertension

Ken Chen,* Chunjiang Fu,* Caiyu Chen,* Li Liu,* Hongmei Ren, Yu Han, Jian Yang, Duofen He, Lin Zhou, Zhiwei Yang, Lianfeng Zhang, Pedro A. Jose, Chunyu Zeng

Abstract—G-protein–coupled receptor kinase 4 (GRK4) gene variants, via impairment of renal dopamine receptor and enhancement of renin–angiotensin system functions, cause sodium retention and increase blood pressure. Whether GRK4 and the angiotensin type 1 receptor (\( AT_1R \)) interact in the aorta is not known. We report that GRK4 is expressed in vascular smooth muscle cells of the aorta. Heterologous expression of the GRK4\( \gamma \) variant 142V in A10 cells increased \( AT_1R \) protein expression and \( AT_1R \)-mediated increase in intracellular calcium concentration. The increase in \( AT_1R \) expression was related to an increase in \( AT_1R \) mRNA expression via the NF-\( \kappa B \) pathway. As compared with control, cells expressing GRK4\( \gamma \) 142V had greater NF-\( \kappa B \) activity with more NF-\( \kappa B \) bound to the \( AT_1R \) promoter. The increased \( AT_1R \) expression in cells expressing GRK4\( \gamma \) 142V was also associated with decreased \( AT_1R \) degradation, which may be ascribed to lower \( AT_1R \) phosphorylation. There was a direct interaction between GRK4\( \gamma \) and \( AT_1R \) that was decreased by GRK4\( \gamma \) 142V. The regulation of \( AT_1R \) expression by GRK4\( \gamma \) 142V in A10 cells was confirmed in GRK4\( \gamma \) 142V transgenic mice; \( AT_1R \) expression was higher in the aorta of GRK4\( \gamma \) 142V transgenic mice than control GRK4\( \gamma \) wild-type mice. Angiotensin II–mediated vasoconstriction of the aorta was also higher in GRK4\( \gamma \) 142V than in wild-type transgenic mice. This study provides a mechanism by which GRK4, via regulation of arterial \( AT_1R \) expression and function, participates in the pathogenesis of conduit vessel abnormalities in hypertension. (Hypertension. 2014;63:00-00.)

Key Words: arteries \( \bullet \) G-protein–coupled receptor kinase 4 \( \bullet \) hypertension \( \bullet \) receptor, angiotensin, type 1

Essential hypertension, which affects 25% of the middle-aged adult population, constitutes a major risk factor for stroke, myocardial infarction, and heart and kidney failure. The kidney, vasculature, and nervous system govern the long-term control of blood pressure by regulating sodium homeostasis, peripheral resistance, and central arterial stiffness; they, in turn, are influenced by numerous hormones and neural and humoral factors. Hypertension may be caused not only by increased activity of prohypertensive systems but also by defects in antihypertensive systems that serve as counter-regulatory mechanisms. Most hormones and humoral factors regulate blood pressure via their receptors, including G-protein–coupled receptors. G-protein–coupled receptors constitute the largest family of cell surface receptors; abnormal G-protein–coupled receptor kinase (GRK) function has the potential to affect receptor-regulated biological responses in many physiological and pathological conditions, including hypertension.

The GRK family plays an important role in the regulation of blood pressure. GRK4 is distinguished from other members of the GRK family by its constitutive activity and limited tissue expression. The GRK4 variants 65 L, 142V, and 486V are associated with essential hypertension in ethnically distinct populations. Overexpression of human (h) GRK4\( \gamma \) 142V or hGRK4\( \gamma \) 486V in mice produces hypertension. The hypertension of spontaneously hypertensive rats may also be explained, in part, by increased renal GRK4 expression. Our previous study found that increased renal GRK4 expression causes the attenuated renal \( D_1 \) dopamine receptor–mediated natriuresis and diuresis that play a role in the pathogenesis of the hypertension in spontaneously hypertensive rats. Increased activity of the renin–angiotensin system is important in the pathogenesis of hypertension. GRK4 interacts not only with the dopaminergic but also with the renin–angiotensin system to regulate blood pressure. Increased renal expressions of both GRK4 and angiotensin type 1 receptor (\( AT_1R \)) contribute to the increased blood pressure in spontaneously hypertensive rats because selective renal silencing of both GRK4 and \( AT_1R \) increases sodium excretion and decreases blood pressure to a greater extent than silencing of either GRK4 or \( AT_1R \).
Conduit and resistance arterial vessels are important in the regulation of blood pressure and myocardial function. Increased aortic stiffness, a risk factor in cardiovascular disease, may be related to increased activity of the renin–angiotensin system. Whether GRK4 and the AT1R interact in the aorta and other arteries in regulating vascular smooth muscle function is not known. Our present study found expression of GRK4 in the tunica media of arteries; vascular smooth muscle cells, transduced with the GRK4γ variant 142V, increased AT1R expression and function. The regulation of AT1R by GRK4 is of physiological significance because AT1R expression and angiotensin II (Ang II)–mediated vasoconstriction in the aorta were greater in hGRK4γ 142V than in hGRK4γ wild-type (WT) transgenic mice. Infusion of the AT1R antagonist, candesartan, lowered blood pressure to a greater and longer extent in hGRK4γ 142V than in hGRK4γ WT transgenic mice. Our present study provides a mechanism by which GRK4, via regulation of arterial AT1R expression and function, participates in the pathogenesis of hypertension.

Methods

Transgenic Mice
hGRK4γ WT and hGRK4γ 142V transgenic mice were generated as previously described in the online-only Data Supplement. As previously reported, the genetic variation is GCC to GTC (amino acid 142V). The transgenic mice were used after 10 weeks of age.

This study was approved by the Third Military Medical University Animal Use and Care Committee. All experiments conformed to the guidelines of the ethical use of animals, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Cell Culture and GRK4 Transduction
Embryonic thoracic aortic smooth muscle cells (passage 10–20) from normotensive Berlin-Druckrey IX (A10; CRL 1476, ATCC) were homogenized in ice-cold lysis buffer (5 mL/g tissue), sonicated, kept on ice for 1 hour, and centrifuged at 16 000g for 30 minutes. All samples were stored at −70°C until use.

The lentivirus-based pLenti6.3-GRK4γ-IRE52-EGFP plasmid (Invitrogen Life Technologies Corporation, Shanghai, China; Figure S2A) was transiently transduced into 293TN cells. The A10 cells (1.5×10⁶/mL) were cultured in 2-mL DMEM medium containing 2% FCS (PAA Laboratories). The A10 cells were infected by polybrene and virus (multiplicity of infection=100). The medium was replaced 48 hours after transduction, and then 5 μg/mL blasticidin was added and incubated for another 48 hours. The transduced cells were identified by green fluorescent protein expression (Figure S2B).

Small Interfering RNA
Small interfering RNA (siRNA) against GRK4 mRNA and its control scrambled RNA were synthesized and purified with reverse-phase high-performance liquid chromatography as 25-mer phosphorothioate-modified oligodeoxynucleotides (GRK4 siRNA sequence: #1 5′-AUCUAAGAGGUGCAUAGAUUCUdTdT-3′, #2 5′-AAGGACCUCAAUGAAUAUAGAAdTdT-3′, #3 5′-TGCAATGACACAATACACGTdTdT-3′, from nucleotides 421 to 436 and 1752 to 1776 of the rat GRK4 cDNA). The effects of 50 nmol/L siRNA were compared with scrambled RNA (control). Briefly, cells were grown in 6-well plates until 60% confluence, and 50 nmol/L siRNA or control RNA was mixed with 6 μL of Oligofectamine in OptiMEM medium (Invitrogen Life Technologies) and incubated for 24 hours, then switched to growth medium and incubated for another 24 hours. The cells were collected and processed for reverse transcription-PCR (RT-PCR) for GRK4 to determine the efficiency of siRNA-induced GRK4 gene silencing (Figure S3).

Immunoblotting
After subjecting the cell lysates to centrifugation at 12000g for 15 minutes, the supernatants of A10 cells were collected and their protein concentrations were measured using a bicinchoninic acid protein assay kit (Hyclone Pierce, Logan, UT). Immunoblotting was performed as previously reported except that the transblots were probed with the rabbit anti-GRK4 antibody (1:400) and rabbit anti-AT1R antibody (1:500; Santa Cruz Biotechnology, CA). The amount of protein transferred onto the membranes was verified by immunoblotting for β-actin.

Confocal Microscopy of Double-Stained Transduced A10 Cells and Artery
The aortae from Sprague-Dawley rats, cleared of blood with ice-cold oxygenated saline and kept in Histochoice (Amresco, Solon, OH) for 1 to 2 days at 4°C, were sectioned (4 μm) and mounted on slides. Reactions with antibodies were performed as described previously in the online-only Data Supplement.

Transduced A10 cells, grown on coverslips, were fixed and permeabilized with 100% methanol (30 minutes). Reactions with antibodies were performed as described previously in the online-only Data Supplement.

Immunoprecipitation
Equal amounts of cell lysates (300 μg protein/mL supernatant) were incubated with affinity-purified anti-GRK4 receptor antibody (3 μL/mL; GRK4/AT1R communoprecipitation) or polyclonal anti-phosphoserine antibody (Zymed Laboratory, San Francisco, CA; AT1R phosphorylation; 1 μg/mL) for 1 hour and protein-G agarose at 4°C for 12 hours. The immunoprecipitates were subjected to immunoblotting with the AT1R antibody. To determine the specificity of the bands found on the immunoblots, IgG (negative control) and AT1R antibody (positive control) were used as the immunoprecipitants, instead of the GRK4 antibody.

RT-PCR of GRK4 and AT1R
A total of 2 μg of total RNA extracted from hGRK4γ WT–transduced cells or hGRK4γ 142V–transduced cells was used to synthesize cDNA and served as a template for amplification of AT1R GRK4, and β-actin, which served as the house-keeping gene control. The AT1R and GRK4 mRNA expressions were normalized by β-actin mRNA.

The GRK4 bands, cut from the gels, were extracted by DNA gel extraction kit (Omega). After purification, the DNA was sequenced and aligned by DNAMAN software (Lynnon Biosoft).

Electrophoretic Mobility Shift Assay
Electrophoretic Mobility Shift Assay (EMSA) was performed with the Light-shift Chemiluminescent EMSA Kit (Pierce Chemical Co, Rockford, IL) according to the manufacturer’s recommendations. A synthetic DNA double-stranded oligonucleotide probe (5′-biotin-AGTTGAGGGGACCTTCCAGGC-5′) containing the sequence of the rat AT1R gene promoter between nucleotides −350 bp and −363 bp (5′-AAGGGAGTTCCCTA-3′) was labeled with biotin and incubated with the nuclear extracts.

Intracellular Calcium Measurement
Intracellular calcium was measured, as previously described with some modifications in the online-only Data Supplement. The free Ca²⁺ concentration was calculated from the equation: [Ca]in = Kd[(R−Rm)(Rm−R)/(Fmax−Fm)]/[Fm−Fm(1−Rm)]. The Kd is the dissociation constant of Fura-2 to calcium. R is the ratio of each 340 nm/380 nm. Minimum and maximum are the fluorescence values of cells treated with Triton X-100 (saturating Ca²⁺ concentration) or by EGTA (ethylenebis(oxyethylenenitrilo) tetraacetic acid; zero Ca²⁺ concentration).

Artery Ring Study
Thoracic aortae were obtained from the hGRK4γ WT and hGRK4γ 142V transgenic mice. Each artery was cut into a ring of 2- to 3-mm long for the experiments, which was used to measure the vascular reactivity to Ang II (Sigma-Aldrich, St. Louis, MO), in the presence or absence of the endothelium, as described in the online-only Data Supplement.
An intact functional endothelium in all preparations was assessed by determining a vasodilatory response to acetylcholine (Ach; 10⁻⁶ mol/L; Sigma). If Ach (10⁻⁶ mol/L) induced the relaxation of artery rings preconstricted with norepinephrine (10⁻⁶ mol/L) by >75%, the arterial endothelium can be considered intact.³³

**Statistical Analysis**

The data are expressed as mean±SEM. Comparison within groups was made by repeated measures ANOVA (or paired t test when only 2 groups were compared), and comparison among groups (or t test when only 2 groups were compared) was made by factorial ANOVA with Holm–Sidak test. A value of P<0.05 was considered significant.

**Results**

**Expression of GRK4 in Artery**

We first determined whether GRK4 is expressed in the aorta by immunofluorescence, immunoblotting, and RT-PCR. Immunofluorescence microscopy showed GRK4 staining in the tunica media and adventitia of aortae from Sprague-Dawley rats and C57BL/6J mice (Figure 1A). GRK4 expression was also found with immunoblotting; specific GRK4 (54, 60, and 65 kDa) bands were found in A10 cells, which were attenuated, especially the 60-kDa band, after transduction with the specific GRK4 siRNA (Figure 1B). The specificity of this GRK4 antibody was reported in our published study.¹⁴ RT-PCR showed the expected 125-bp GRK4 band, based on the primers, which was not observed when RNA was omitted in the RT period (Figure 1C). The gel containing the 125-bp band was cut, sequenced, and aligned by DNAMAN software (Lynnon Biosoft; Figure S4).

To confirm the GRK4 expression in the adventitia, we checked the GRK4 expression in fibroblasts and adipocytes by immunoblotting and RT-PCR. We found that both fibroblasts and adipocytes expressed GRK4 (Figure 1Da and 1Db). Removal of the adventitia did not affect the Ang II–mediated vasoconstriction, indicating that the GRK4 in the adventitia did not participate in the Ang II–mediated vasoconstriction (Figure 1Dc). The physiological significance of GRK4 in the adventitia remains to be determined.

We measured the GRK4 expression in large and small vessels, including the thoracic aorta, superior mesenteric artery, carotid arteries, and renal artery, and found there was no difference for the GRK4 expression in those vessels (Figure S5).

**Regulation by GRK4 of AT₁R Expression and Function in A10 Cells**

AT₁R antibody specificity was determined by immunoblotting, the 43-kDa band was absent in the aorta from AT₁R⁻/⁻ mice and no longer visible in A10 and renal proximal tubule.
cells (positive control) when the antibody was preadsorbed with the immunizing peptide (Figure 2A).

To determine the effect of hGRK4 on AT1R expression, we used A10 cells transduced with hGRK4γ 142V. We found that the GRK4 expression was not different between hGRK4γ 142V and control (GRK4γ WT) cells (Figure S6). However, AT1R protein and mRNA expressions were higher in hGRK4γ 142V than in GRK4γ WT cells (Figure 2B and 2C); AT1R protein degradation was lower in hGRK4γ 142V–transduced cells than in GRK4γ WT–transduced cells (Figure 2D), indicating that the regulation of AT1R expression by hGRK4γ occurred at both post-translational and transcriptional levels. In addition, AT1R phosphorylation was lower in hGRK4γ 142V–transduced cells than in hGRK4 WT–transduced cells (Figure 2E), indicating that the decreased AT1R protein degradation may be ascribed to decreased AT1R phosphorylation. The increased AT1R expression is physiologically relevant because the intracellular calcium concentration after stimulation with Ang II (10−7 mol/L) was higher in hGRK4γ 142V–transduced cells than in hGRK4 γ WT–transduced cells (Figure 3).

To investigate whether Ang II was involved in the regulation of GRK4 on AT1R expression, we measured the concentration of Ang II in the A10 cell culture supernatant and cell lysate; Ang II concentrations were not different between hGRK4γ 142V–transduced cells and hGRK4γ WT–transduced cells (culture supernatant: 113.87±13.07 versus 108.73±12.76; cell lysate: 237.3±23.7 versus 217±20; n=5; P=NS). The angiotensin-converting enzyme inhibitor, captopril (10−4 mol/L, Sigma-Aldrich, St. Louis, MO), also had no effect on the AT1R expression in both cell types (Figure S7).

As a regulator of AT1R promoter activity, we measured nuclear factor-xB (NF-xB) binding to the AT1R promoter and found it higher in hGRK4γ 142V–transduced cells than in hGRK4γ WT–transduced cells (Figure 4A). Blockade of NF-xB with an NF-xB inhibitor, BAY11-7082, inhibited the increase in AT1R expression in hGRK4γ 142V–transduced cells (Figure 4B), indicating that NF-xB was involved in the positive regulation of AT1R expression by hGRK4γ 142V.

As aforementioned, the decreased AT1R degradation could be ascribed to the decrease in AT1R phosphorylation in GRK4γ

![Figure 2. Expression of angiotensin type 1 receptor (AT1R) in hGRK4γ wild-type (WT)–transduced and hGRK4γ 142V–transduced A10 cells. A, Specificity of AT1R antibody. Protein (100 μg) from A10 cells, renal proximal tubule (RPT) cells from Wistar-Kyoto rats, and aortae from AT1R−/− mice were subjected to immunoblotting with anti-AT1R antibody (1:500) with or without preincubation with the AT1R antibody immunizing peptide (Santa Cruz, 1:10 wt/wt incubation for 12 hours). These studies were repeated ≥3×. B and C, AT1R protein (B; n=7) and mRNA (C; n=6) expressions in hGRK4γ WT–transduced and hGRK4γ 142V–transduced A10 cells. Results are expressed as the ratio of AT1R receptor and β-actin (*P<0.05 vs WT). D, AT1R protein degradation in hGRK4γ WT–transduced and hGRK4γ 142V–transduced A10 cells. The cells were incubated with cycloheximide (10−5 mol/L) for the indicated times. Results are expressed as percentage change of control in each group (n=8; *P<0.05 vs WT). E, AT1R phosphorylation in hGRK4γ WT–transduced and hGRK4γ 142V–transduced A10 cells. The A10 cell lysate protein was immunoprecipitated with anti-phosphoserine antibody and immunoblotted with anti-AT1R antibody (n=3; *P<0.05 vs WT). GRK4 indicates G-protein–coupled receptor kinase 4.](http://hyper.ahajournals.org/content/early/2014/07/10/HYPERTENSIONAHA.114.013731)
142V–transduced cells. An additional study found a colocalization (Figure 5A) and coimmunoprecipitation (Figure 5B) between GRK4 and AT1R; the coimmunoprecipitation of GRK4 and AT1R was less in hGRK4γ142V–transduced cells than in hGRK4γWT–transduced cells (Figure 5B), which could be a factor in the decreased phosphorylation of AT1R in hGRK4γ142V–transduced cells.

AT1R Expression and Function in hGRK4γ142V Transgenic Mice

To further investigate the physiological role of the GRK4-regulated AT1R expression, we studied AT1R expression and function in hGRK4γWT and hGRK4γ142V transgenic mice. Consistent with previous reports,6,8,11,13 anesthetized hGRK4γ142V transgenic mice had higher systolic, diastolic, and mean blood pressures (systolic blood pressure=123.37±8.19, diastolic blood pressure=96.37±4.78 mmHg, mean blood pressure=104.54±3.99; n=11) than anesthetized hGRK4γWT transgenic mice (systolic blood pressure=98.38±5.42, diastolic blood pressure=83.00±4.54 mmHg, mean blood pressure=88.21±3.63; n=11; P<0.001). Although GRK4 expression was not different between hGRK4γWT 142V and hGRK4γ142V transgenic mice (Figure S5), AT1R expression in aorta was higher in hGRK4γ142V than in hGRK4γWT transgenic mice (Figure 6A). We also studied the vasoconstrictor effect of Ang II on the aorta from hGRK4γ142V and hGRK4γWT transgenic mice. The vasoconstriction caused by Ang II was greater in hGRK4γ142V than in hGRK4γWT transgenic mice in the presence or absence of the endothelium. The AT1R blocker, candesartan (10−6 mol/L), blocked the vasoconstrictor effect of Ang II, in both transgenic mice such that there was no longer any difference between the 2 mouse strains (Figure 6B).

Figure 3. Intracellular calcium concentration in hGRK4γ wild-type (WT)–transduced and hGRK4γ142V–transduced A10 cells. Representative tracing of the effect of angiotensin II (Ang II; 10−7 mol/L) on intracellular free calcium in hGRK4γ WT–transduced and hGRK4γ 142V–transduced A10 cells. Ang II was added 15 s after the start of the experiment, shown as the arrow in the figure (n=8). GRK4 indicates G-protein–coupled receptor kinase 4.

Figure 4. Role of nuclear factor-κB (NF-κB) on angiotensin type 1 receptor (AT1R) expression in GRK4γ wild-type (WT)–transduced and hGRK4γ142V–transduced A10 cells. A, Electrophoretic Mobility Shift Assay (EMSA) of nuclear protein from A10 cells. Binding activity of AT1R gene promoter (−350 bp and−363 bp), containing an NF-κB site, was examined in nuclear proteins from hGRK4γ WT–transduced (lane 2) and hGRK4γ142V (lane 3)–transduced A10 cells by EMSA. No nuclear extracts (lane 1) or 50× unlabeled probe (lane 4) was added to the reaction mixture and served as negative controls. B, Effect of NF-κB on GRK4-mediated regulation of AT1R protein expression in A10 cells. hGRK4γ WT–transduced or hGRK4γ142V–transduced cells were treated with or without the NF-κB inhibitor BAY11-7082 (20 μmol/L) for 24 hours. Results are expressed as the ratio of AT1R and β-actin (n=5, *P<0.05 vs vehicle).

AT1R expression in the aorta of hGRK4γ WT and hGRK4γ142V transgenic mice (Figure S5). We also showed that the expression of AT1R was greater in hGRK4γ142V than in hGRK4γ WT transgenic mice in the presence or absence of the endothelium. The AT1R blocker, candesartan (10−6 mol/L), blocked the vasoconstrictor effect of Ang II, in both transgenic mice such that there was no longer any difference between the 2 mouse strains (Figure 6B).

Consistent with a previous report,34 the intravenous infusion of 10 μL/h caused a greater decrease in blood pressure in hGRK4γ142V than in hGRK4γ WT transgenic mice (Figure S8).

Discussion

GRK4, as with the other members of the GRK family, is predominantly localized at the plasma membrane, as a result of palmitoylation of its C-terminal cysteine residues.35 GRK4 differs from the other GRKs in tissue distribution; GRKs 2, 3, 5, and 6 are ubiquitously expressed, whereas GRK4 is abundantly expressed in the testis, myometrium, and kidney.7,8,11 We now show for the first time the expression of GRK4 in the aorta, determined by immunoblotting, immunohistochemistry (tunica media), and RT-PCR, implying that GRK4 could be involved in the regulation of vascular smooth muscle function.
There is increasing evidence that GRK4 plays an important role in the pathogenesis of hypertension.6–8,11,12,16,36 The GRK4 locus (4p16.3) is linked to and GRK4 gene variants are associated with human essential hypertension.6–11,12,36–38 In Ghanaians, the 2-locus model of angiotensin-converting enzyme I/D and GRK4 gene variants are associated with hypertension in African whites.36,37 GRK4 variants, including 65L, 142V, and 486V, by themselves, or interaction with other variants of other genes are associated with hypertension in American whites,39 Australian whites,12 Italians,36 and northern Han Chinese.40 We have reported that hGRK4γ 142V transgenic mice on 98% C57BL/6J and SJL/J background. C57BL/6 mice are salt-sensitive, whereas SJL/J mice are salt-resistant.41 We now report that hGRK4γ 142V transgenic mice on mixed C57BL/6J and SJL/J background have increased blood pressure.

We have reported that hGRK4γ 142V transgenic mice have increased blood pressure and impaired ability to excrete a sodium load.41 The impaired sodium excretion is mainly because of a dysfunction of the D1 dopamine receptor46–8,11,14,42 Dopamine, produced by the renal proximal tubule, is important in the regulation of sodium excretion and blood pressure.4,6–8,11,14,42 Although the renal dopaminergic system keeps the blood pressure from increasing after a moderate sodium load,4,6–8,11,14,42 the renin–angiotensin system, including the AT1R, is crucial in sodium retention and maintenance of blood pressure, especially under conditions of sodium deficit.5,8,15 Both GRK4- and AT1R exist in vascular smooth muscle cells, but whether GRK4-mediated regulation of blood pressure involves the AT1R in vascular smooth muscle cells is not known. Our present study found that compared with hGRK4γ WT transgenic mice, hGRK4γ 142V transgenic mice have higher arterial AT1R expression and Ang II–mediated aortic vasoconstriction. Ang II–mediated increase in intracellular calcium is also increased to a greater extent in hGRK4γ 142V–transduced than in hGRK4γ WT–transduced A10 aortic cells. The stimulatory effect of hGRK4γ 142V on AT1R receptor expression and function is physiologically relevant because the intravenous infusion of Ang II increased, whereas the intravenous infusion of an AT1R antagonist, candesartan, decreased blood pressure to a greater degree and longer extent in hGRK4γ 142V than in hGRK4γ WT transgenic mice. In the current study, the transgenic mice are on 50% C57BL/6 Jackson and 50% SJL Jackson mouse background. GRK4 and AT1R protein expression are greater in C57BL/6 Jackson than in SJL Jackson mice.41 hGRK4γ 142V transgenic mice on C57BL/6 background are also hypertensive that is caused, in part, by decreased renal D1 receptor function41,13,34 and increased renal AT1R expression.42 The increase in blood pressure in hGRK4γ 142V in C57BL/6 and SJL Jackson mice is not mitigated by the 50% SJL Jackson genetic background and is thus independent of the presence of the salt-resistant phenotype.

As a kinase, GRK4 phosphorylates ligand-occupied and ligand-occupied G-protein–coupled receptors as their primary substrates, such as the D1 dopamine receptor.6–11 Increased GRK4 activity augments D1 receptor phosphorylation in kidney.6,8,10,11,14 However, our present study found that increased GRK4 activity decreases AT1R phosphorylation, which seems counterintuitive, at first glance. Our experiments uncover a possible mechanism: there is a linkage between GRK4 and AT1R in vascular smooth muscle cells, and it is interesting to find that the GRK4/AT1R linkage is decreased in A10 cells transduced with hGRK4γ 142V, which may therefore cause decrease in AT1R phosphorylation in the hGRK4γ 142V A10–transduced cells. The decreased phosphorylation of AT1R in hGRK4γ 142V A10–transduced cells may be involved in the hGRK4γ 142V–mediated upregulation of AT1R expression because in the present study, we found that a decreased AT1R degradation accompanies the decreased AT1R phosphorylation in hGRK4γ 142V–transduced A10 cells. The pathway leading to the lower
binding of hGRK4γ 142V with AT1R receptor is not known, which needs to be elucidated in the future.

The regulation of hGRK4γ on AT1R expression is complicated, as in our previous study, we found that in addition to hGRK4γ 142V–mediated decrease in AT1R degradation, AT1R transcription is also increased, as evidenced by increased AT1R mRNA in hGRK4γ 142V–transduced A10 cells. The activity of NF-κB, a regulator of AT1R promoter activity, is increased, accompanied by an increase in its binding to the AT1R promoter in hGRK4γ 142V–transduced A10 cells. In the presence of an NF-κB inhibitor, the increase in AT1R expression in hGRK4γ 142V–transduced A10 cells is abolished, confirming the important role of NF-κB in this process.

Conclusions and Perspectives

Our previous study found that increased renal GRK4 expression causes the attenuated renal D1 dopamine receptor–mediated natriuresis and diuresis and increased renal AT1R-mediated sodium excretion that play a role in the pathogenesis of the hypertension in spontaneously hypertensive rats.15,16 The present study reinforces the role of GRK4 in hypertension and shows that a constitutively increased activity of GRK4 increases arterial AT1R receptor expression and function, which may be involved in the abnormalities of conduit vessels in essential hypertension. The results imply that the inhibition of GRK4 expression or activity, based on the chemical or biological medicine, may be an effective therapeutic approach for essential hypertension.

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Disclosures

Dr. Jose, who is the Scientific Director of Hypogen, Inc, owns US Patent Number 6,660,474 for G protein–related kinase mutants in essential hypertension. The other authors report no conflicts.

References

The gene variant of G-protein-coupled receptor kinase 4 (GRK4), GRK4γ 142V, is associated with hypertension. Our previous study found that increased renal GRK4 activity attenuated renal D, dopamine receptor and increased renal angiotensin type 1 receptor (AT1R) functions. In these studies, we report for the first time that GRK4γ is expressed in vascular smooth muscle cells of the aorta and GRK4γ142V decreased AT1R degradation, via decreased phosphorylation and increased AT1R expression, via NF-κB. In A10 cells, expression of GRK4γ142V augmented the angiotensin II-mediated increase in intracellular Ca2+ levels. In transgenic mice on novel C57Bl/6J and C57BL/6J background, angiotensin II-induced vasoconstriction was increased in the aorta from GRK4γ142V transgenic mice, compared with GRK4γ wild-type transgenic mice. Finally, the hypertension in GRK4γ142V transgenic mice was related to an increase in angiotensin II-mediated vasoconstriction.

The present study reinforces the role of GRK4 in hypertension and shows that a constitutively increased activity of GRK4 increases arterial AT1R receptor expression and function, which may be involved in the abnormalities of conduit vessels in essential hypertension. The results imply that the inhibition of GRK4 expression or activity, based on the chemical or biological medicine, may be an effective therapeutic approach for essential hypertension.
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Role of GRK4 in the Regulation of Arterial AT1 Receptor in Hypertension

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Supplementary Methods

Generation of transgenic mice
Two constructs were used to generate the transgenic mice. The full-length WT hGRK4γ cDNA was obtained by PCR by using the GRK4γ cDNA in pTRE plasmid as the template. The 142V polymorphism was generated using site-directed mutagenesis. The two cDNAs were subcloned into pcDNA3.1. Expression of the cDNA insert was under the control of the cytomegalovirus promoter and bovine growth hormone (BGH) poly (A) signal. Full-length cDNA was verified by sequencing. The GRK4γ transgenic mice were generated by microinjecting the cDNA constructs into fertilized eggs obtained from the mating of a C57BL/6J F1 female mouse and a SJL/J F1 male mouse at the Institute of Animal Laboratory Science, Chinese Academy of Medical Sciences, Comparative Medicine Center, Peking Union Medical College. The presence of the transgene in the transgenic mice was verified by PCR and sequencing studies. The DNA samples were obtained from toes or tails of the hGRK4γ WT or hGRK4γ 142V transgenic mice. The forward primer of hGRK4γ was 5’-GATGAGGACCGAAGTGATTGT-3’ and the reverse primer was 5’-TTGCCCAGGTTGTAAATGTG-3’ (GenBank accession no. NM_001004057, 560bp). The amplification was performed with the following conditions: 35 cycles of denaturation at 95°C for 2 min, annealing for 30 sec at 58°C, and extension for 40 sec at 72°C. The PCR products were sequenced using the Applied Biosystem 377 DNA sequencer (Perkin Elmer, Wellesley, MA) and compared with the NCBI sequence (NM_001004057).

Arterial sample of AT1R knock-out mice
AT1R knock-out (AT1R-/-) mice in this study were obtained from laboratory of Dr Yanfang Chen (Wright State University, Dayton, OH, US). The genotype was determined by PCR analysis of genomic DNA which isolated from mice tail tissue according to previously reported methods. The artery tissues were isolated and washed with ice-cold PBS for several times, and lysed by homogenizer in ice-cold lysis buffer. After centrifugation, the supernatant was mixed in 6×sample buffer and boiled for 5 min, stored at -70°C until use for immunoblotting.

Cell culture
Immortalized RPT cells from WKY were cultured at 37°C in 95% air and 5% CO2 atmosphere in FBS% DMEM/F-12 culture media, as previously described. 3T3-L1 preadipocytes, obtained from the American Type Culture Collection (CL-173, ATCC, Manassas, VA), are confirmed as a cell line of mouse preadipocytes. The cells were cultured in high-glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum in 5% CO2 humidified atmosphere at 37°C. The differentiation of adipocytes was induced according to
previously reported methods.\(^6\) Rat2 cells were obtained from the American Type Culture Collection (CL-1764, ATCC, Manassas, VA), which are confirmed as a cell line of rat fibroblast\(^7\).

The cells (80% confluence) were extracted in ice-cold lysis buffer, sonicated, kept on ice for 1hr, and centrifuged. The supernatants were used for immunoblotting.

**Confocal microscopy of double-stained transfected A10 cells and artery**
For the aorta, GRK4 was visualized using the goat anti-GRK4 antibody (1:400) followed by a fluorescein isothiocyanate (FITC); α-smooth muscle actin was visualized using rabbit anti-smooth muscle actin antibody (1:50-1:100), followed by a rhodamine (TRITC)-conjugated affinity-purified goat anti-rabbit secondary antibody (red; Molecular Probes).

For the transduced A10 cells, GRK4 (1:100) was visualized using a monoclonal mouse anti-GRK4 receptor antibody (Abcam, Cambridge, UK), followed by AMCA-goat anti-mouse IgG antibody (blue; Jackson ImmunoResearch Laboratory, West Grove, PA). AT\(_1\)R was visualized using a polyclonal rabbit anti-AT\(_1\)R antibody (1:100), followed by rhodamine–conjugated goat anti-rabbit IgG antibody (red; Jackson ImmunoResearch Laboratory, West Grove, PA). Cells that were treated with only AMCA-goat anti-mouse IgG antibody or rhodamine-conjugated goat anti-rabbit IgG antibody revealed no immunofluorescence, and omission of the anti-AT\(_1\)R antibody showed no red or purple color after merging the images (data not shown).

**RT-PCR of GRK4 and AT\(_1\)R**
For β-actin, the forward primer was 5’- CCACTGCGCGATCCTCTT -3’ and the reverse primer was 5’-GTCAGCAATGGCCTGGGTA-3’ (GenBank accession no. NM_031144, 251bp). For the AT\(_1\)R, the forward primer was 5’-AAATTGAGTGGCTGTATG-3’ and the reverse primer was 5’-CTTGACCTCCATCTCCTC-3’ (GenBank accession no. NM_031009, 160bp). The amplifications were both performed with the following conditions: 35 cycles of denaturation at 94°C for 2 min, annealing for 30 sec at 59°C, and extension for 45 sec at 72°C. For GRK4, the forward primer was 5’- TGTCCTGATCCTGAGGC-3’ and the reverse primer was 5’-ACACACCCTGTCGCAAAT-3’ (GenBank accession no. NM_022928.1, 125bp). The amplification was performed with the following conditions: 35 cycles of denaturation at 95°C for 2 min, annealing for 30 sec at 59°C, and extension for 45 sec at 72°C.

**Intracellular calcium measurement**
Transduced cells (1×10\(^6\)/ml) were seeded in 60 mm glass dish and incubated with 5µM calcium-dependent fluorescent indicator Fura-2 in DMEM medium at 37°C. The cells were then washed twice with Krebs buffer (140 mM NaCl, 5.4mM KCl, 0.5mM CaCl\(_2\), 1.2mM MgSO\(_4\), 0.3Mm NaH\(_2\)PO\(_4\), 10 mM HEPES, 5mM glucose, pH 7.4 with Tris base). The cells were dispersed by trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA). The cell suspensions (200 µl, 1×10\(^5\)/ml) were transferred into a
96-well cell culture plates (Thermo, Rochester, NY), which were placed in the Thermo Varioskan flash instrument (Thermo Fisher Scientific, Waltham, MA). Fluorescence was detected every 5 sec alternating between 340 and 380 nm excitation (2 nm slit size) at 510 nm emission (5 nm slit size).

Aorta ring study
Aorta rings were mounted between a post- and a force-transducer (AD Instruments, Sydney, Australia) that was attached to a micrometer, then immersed into a 10 ml isolated organ chamber (Scientific Instruments, Barcelona, Spain) containing Krebs-Henseleit solution (K-H solution; in mM): 118 NaCl, 4.7 KCl, 25 NaHCO₃, 1.03 KH₂PO₄, 0.45 MgSO₄•7H₂O, 2.5 CaCl₂, and 11.1 glucose at pH 7.35 to 7.45, which was continuously bubbled with 95% O₂/5% CO₂ gas mixture, and the temperature was maintained at 37°C. Preload tension was 0.5g, and the K-H solution was replaced every 20 min. After a 2-hr equilibration, Ang II (final concentrations, 10⁻⁸-10⁻⁵M) was added into the organ chamber with or without a 15 min-candesartan (10⁻⁶M) pre-incubation. The tension of the aorta rings was recorded and expressed by change in amplitude from baseline.

Figure S1. Expression of hGRK4 $\gamma$ WT and hGRK4 $\gamma$ 142V in mice, verified by gene sequencing. The variant nucleotide is indicated in the figure.
**Figure S2.** Transduction of hGRK4 γ WT and hGRK4 γ 142V in A10 cells

**A:** The constructs of pLenti6.3-hGRK4 γ -IRES2-EGFP plasmids. **B:** GFP expression in hGRK4 γ -transfected cells: GFP expression (green) was evident in A10 cells transduced with hGRK4 γ WT and hGRK4 γ 142V A10 cells.
**Figure S3.** Effect of GRK4 siRNA on GRK4 mRNA expression. GRK4 RT-PCR products from testis (positive control), aorta, and A10 cells (control, siRNA#1, siRNA#2, and scrambled RNA) were analyzed in 10% polyacrylamide gel stained with ethidium bromide. An amplification product of the predicted size (125 bp) is seen in RT-PCR reaction using RNA (1 μg). The band was attenuated after transfection with the specific GRK4 siRNA into A10 cells (GRK4 siRNA sequence: #1: 5’-AUCUAAAGAGGGAGGCAUUGAAUUCUUDdTdT-3’; #2: 5’-AAGGACCCUCAUGAAUAUGAAGAUAdTdT-3’) compared with the band of A10 cells without siRNA transfection (scrambled RNA sequence: 5’-TGACGATAAGAACAATAACdTdT-3’).
Figure S4. Sequencing of 125 bp GRK4 RT-PCR product. The RT-PCR products were identified by PAGE; the 125bp GRK4 bands were cut, extracted (by DNA gel extraction kit, Omega, US), and sequenced. The sequence of the PCR product almost completely aligned with the sequence of GRK4 (NM_022928.1).
Figure S5. A: Expression of GRK4 in hGRK4 γ WT and 142V transgenic mice. Results are expressed as the ratio of GRK4 and β-actin (n=4, P=NS); B: Expression of GRK4 in large and small arterial vessels. Results are expressed as the ratio of GRK4 and β-actin (n=4, P=NS).
Figure S6. Expression of GRK4 in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells. Results are expressed as the ratio of GRK4 and β-actin (n=4, P=NS)
Figure S7. Effect of the angiotensin converting enzyme inhibitor (ACEI), captopril, on AT₁R expression in hGRK4 γ WT- and hGRK4 γ 142V-transduced A10 cells. Results are expressed as the ratio of AT₁R and β -actin (n=4, P=NS).
Figure S8. Effect of Ang II and the AT₁R antagonist, candesartan, on systolic blood pressure (BP) in hGRK4 γ WT and hGRK4 γ 142V transgenic mice. Ang II (1 mg/kg/min at the rate of 10 μl/hr) increased systolic BP to a greater extent in hGRK4 γ A142V than hGRK4 γ WT transgenic mice. Conversely, an intravenous administration of candesartan (0.139 mg/kg/min at the rate of 10 μl/hr) by mini-pump decreased systolic BP to a greater extent in hGRK4 γ 142V than hGRK4 γ WT transgenic mice. The mice were anesthetized with pentobarbital and BPs measured from the femoral artery. BPs were obtained after a 1-hr stabilization period (n =11, *P<0.001 vs. WT mice).