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Role of GRK4 in the Regulation of Arterial AT_1 Receptor in Hypertension

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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 or not 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 (VSMCs) of the aorta. Heterologous expression of the GRK4γ 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-κB pathway. As compared with control, cells expressing GRK4γ 142V had greater NF-κB activity with more NF-κB bound to the AT_1R promoter. The increased AT_1R expression in cells expressing GRK4γ 142V was also associated with decreased AT_1R degradation, which may be ascribed to lower AT_1R phosphorylation. There was a direct interaction between GRK4γ wild-type (WT) and AT_1R that was decreased by GRK4γ 142V. The regulation of AT_1R expression by GRK4γ 142V in A10 cells was confirmed in GRK4γ 142V transgenic mice; AT_1R expression was higher in the aorta of GRK4γ 142V transgenic mice than control GRK4γ wild-type (WT) mice. Angiotensin II-mediated vasoconstriction of the aorta was also higher in GRK4γ 142V than WT 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.

Keywords

GRK4; AT_1R; artery; hypertension

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Disclosures
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Introduction

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 [1, 2]. The kidney, vasculature, and nervous system govern the long-term control of blood pressure by regulating sodium homeostasis, peripheral resistance, and central arterial stiffness [3-5]; they, in turn, are influenced by numerous hormones and neural and humoral factors. Hypertension may be caused not only by increased activity of pro-hypertensive systems but also by defects in anti-hypertensive systems that serve as counter-regulatory mechanisms [4, 6-8]. Most hormones and humoral factors regulate blood pressure via their receptors, including G protein-coupled receptors (GPCRs). GPCRs comprise the largest family of cell surface receptors [6-8]; 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 [4, 5, 7].

The GRK family plays an important role in the regulation of blood pressure[9]. GRK4 is distinguished from other members of the GRK family by its constitutive activity [10, 11] and limited tissue expression [6, 7]. The GRK4 variants 65L, 142V, and 486V are associated with essential hypertension in ethnically distinct populations [8, 11, 12]. Over-expression of human (h) GRK4γ 142V or hGRK4γ 486V in mice produces hypertension [8, 13]. The hypertension of spontaneously hypertensive rats (SHRs) may also be explained, in part, by increased renal GRK4 expression [14]. 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 SHRs[14].

Increased activity of the renin-angiotensin system is important in the pathogenesis of hypertension [5, 15]. GRK4 interacts not only with the dopaminergic but also with the renin-angiotensin system to regulate blood pressure [8]. Increased renal expressions of both GRK4 and angiotensin type 1 receptor (AT_1R) contribute to the increased blood pressure in SHRs 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[16].

Conduit and resistance arterial vessels are important in the regulation of blood pressure and myocardial function[17]. Increased aortic stiffness, a risk factor in cardiovascular disease, may be related to increased activity of the renin-angiotensin system [3, 18]. Whether or not GRK4 and the AT_1R 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 (VSMCs), transduced with the GRK4γ variant 142V, increased AT_1R expression and function. The regulation of AT_1R by GRK4 is of physiological significance because AT_1R expression and angiotensin II (Ang II)-mediated vasoconstriction in the aorta were greater in hGRK4γ 142V than hGRK4γ wild-type (WT) transgenic mice. Infusion of the AT_1R antagonist, candesartan, lowered blood pressure to a greater and longer extent in hGRK4γ 142V than hGRK4γ WT transgenic mice. Our present study provides a mechanism by which GRK4, via regulation of arterial AT_1R expression and function, participates in the pathogenesis of hypertension.

Methods

1. Transgenic mice

hGRK4γ WT and hGRK4γ 142V transgenic mice were generated as previously described[11, 13] in **Supplemental Materials**. As previously reported [11, 12, 19], the genetic variation is GCC to GTC (amino acid 142V, rs1024323). (**Supplementary Figure S1**)

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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.

2. 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/gm tissue), sonicated, kept on ice for 1 hr, and centrifuged at 16,000 g for 30 min. All samples were stored at −70°C until use.

The lentivirus-based pLenti6.3-hGRK4γ-IRES2-EGFP plasmid (Invitrogen Life Technologies Corporation, Shanghai, China) (**Supplementary Figure S2A**), was transiently transduced into 293TN cells. The A10 cells (1.5×10^6 /ml) were cultured in 2 ml DMEM medium containing 2% FBS, 8 μg/ml polybrene and virus (MOI=100). The medium was replaced 48h after transduction, and then 5 μg/ml blasticidin was added and incubated for another 48h. The transduced cells were identified by GFP expression (**Supplementary Figure S2B**).

3. 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'-AUCUAAAGAGGUGCAUUGAAUUCUUdTdT-3', #2 5'-AAGGACCUCAAUGAAUAUGAAGAUAdTdT-3'; scrambled RNA sequence: 5′-TGACGATAAGAACAATAACdTdT-3′), from nucleotides 412 to 436 and 1752 to 1776 of the rat GRK4 cDNA.

The effects of 50 nM siRNA were compared with scrambled RNA (control). Briefly, cells were grown in 6-well plates until 60% confluence, and 50 nM siRNA or control RNA were mixed with 6 μL of oligofectamine in Optimem medium (Invitrogen Life Technologies) and incubated for 24 hr, then switched to growth medium and incubated for another 24 hr. The cells were collected and processed for RT-PCR for GRK4 to determine the efficiency of siRNA-induced *GRK4* gene silencing (**Supplementary Figure S3**).

4. Immunoblotting

After subjecting the cell lysates to centrifugation at 12,000 g for 15 min, the supernatants of A10 cells were collected and their protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Hyclone Pierce, Logan, USA). Immunoblotting was performed as previously reported [20, 21], except that the transblots were probed with the rabbit anti-GRK4 antibody (1:400) and rabbit anti-AT_1R antibody (1:500) (Santa Cruz Biotechnology, CA). The amount of protein transferred onto the membranes was verified by immunoblotting for β-actin.

5. Confocal microscopy of double-stained transduced A10 cells and artery

The aortae from Sprague-Dawley (SD) rats, cleared of blood with ice-cold oxygenated saline and kept in Histochoice (Amresco, Solon, OH) for 1-2 days at 4°C, were sectioned (4 μm), embedded in paraffin, and mounted on slides. Reactions with antibodies were performed as described previously[22-26]. in **Supplemental Materials**

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Transduced A10 cells, grown on coverslips, were fixed and permeabilized with 100% methanol (30 min). Reactions with antibodies were performed as described previously[27] in **Supplemental Materials**.

6. Immunoprecipitation

Equal amounts of cell lysates (300 μg protein/ml supernatant) were incubated with affinity-purified anti-GRK4 receptor antibody (3μl/ml) (GRK4/AT_1R co-immunoprecipitation) or polyclonal antiphosphoserine antibody (Zymed Laboratory, San Francisco, CA) (AT_1R phosphorylation) (1μg/ml) for 1 hr and protein-G agarose at 4°C for 12 hr. The immunoprecipitates were subjected to immunoblotting with the AT_1R antibody. To determine the specificity of the bands found on the immunoblots, IgG (negative control) and AT_1R antibody (positive control) were used as the immunoprecipitants, instead of the GRK4 antibody.

7. RT-PCR of GRK4 and AT_1R

A total of 2 μg of total RNA extracted from hGRK4γ WT or hGRK4γ 142V transduced cells was used to synthesize cDNA and served as a template for amplification of AT_1R, GRK4, and *β*-actin, which served as the house-keeping gene control. The AT_1R 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, US). After purification, the DNA was sequenced and aligned by DNAMAN software (Lynnon Biosoft, USA)

8. Electrophoretic mobility shift assay (EMSA)

EMSA was performed with the Light-shift Chemiluminescent EMSA Kit (Pierce Chemical Co., Rockford, IL), according to the manufacturer's recommendations[28, 29]. A synthetic DNA double-stranded oligonucleotide probe (5'-biotin-AGTTGAGGGGACTTTCCCAGGC-5') containing the sequence of the rat AT_1R gene promoter between nucleotides -350 bp and -363 bp (5'-AAGGGAGTTCCCTA-3') was labeled with biotin and incubated with the nuclear extracts.

9. Intracellular calcium measurement

Intracellular calcium was measured, as previously described with some modifications[30, 31] in **Supplemental Materials**. The free Ca^{2+} concentration $[Ca^{2+}]_{free}$ was calculated from the equation[32]: $[Ca^{2+}]_{free} = Kd[(R-R_{min})/(R_{max}-R)](F_{380max}/F_{380min})$; The *K*d 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 by Triton X-100 (saturating Ca^{2+} concentration) or by EGTA (zero Ca^{2+} concentration).

10. 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 **Supplemental Materials**.

An intact functional endothelium in all preparations was assessed by determining a vasodilatory response to acetylcholine (Ach) (10^{-6} M; Sigma). If Ach (10^{-6} M) induced the relaxation of artery rings preconstricted with norepinephrine (10^{-6} M) by more than 75%, the arterial endothelium can be considered intact [33].

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11. 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

1. Expression of GRK4 in artery

We first determined if 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 SD rats and C57BL/6J mice (**Figure 1A**). GRK4 expression was also found with immunoblotting; specific GRK4 (54 kDa, 60 kDa, 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 125bp band was cut, sequenced, and aligned by DNAMAN software (Lynnon Biosoft, USA) (**Supplemental 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 (**Figures 1D-a and 1D-b**). Removal of the adventitia did not affect the Ang II-mediated vasoconstriction, indicating that the GRK4 in the adventitia did not take participate in the Ang II-mediated vasoconstriction (**Figure 1D-c**). 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 (**supplemental Figure S5**).

2. Regulation by GRK4 of AT_1R expression and function in A10 cells

AT_1R antibody specificity was determined by immunoblotting, the 43kDa band was absence in the aorta from $AT_1R^{-/-}$ mice and no longer visible in A10 and renal proximal tubule cells (positive control) when the antibody was pre-adsorbed with the immunizing peptide (**Figure 2A**).

To determine the effect of hGRK4 on AT_1R expression, we used A10 cells transduced with hGRK4γ 142V. We found, although the GRK4 expression was not different between hGRK4γ 142V and control (GRK4γ WT) cells (**Supplemental Figure S6**). However, AT_1R protein and mRNA expressions were higher in hGRK4γ 142V than GRK4γ WT cells (**Figures 2B and 2C**); AT_1R protein degradation was lower in hGRK4γ 142V- than GRK4γ WT-transduced cells (**Figure 2D**), indicating that the regulation of AT_1R expression by hGRK4γ occurred at both post-translational and transcriptional levels. In addition, AT_1R phosphorylation was lower in hGRK4γ 142V- than hGRK4 WT-transduced cells (**Figure 2E**), indicating that the decreased AT_1R protein degradation may be ascribed to decreased AT_1R phosphorylation. The increased AT_1R expression is physiologically relevant because the intracellular calcium concentration after stimulation with Ang II (10^{-7} M) was higher in hGRK4γ 142V- than hGRK4γ WT-transduced cells (**Figure 3**).

To investigate whether or not Ang II was involved in the regulation of GRK4 on AT_1R expression, we measured the concentration of Ang II in the A10 cell culture supernatant and

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cell lysate; Ang II concentrations were not different between hGRK4γ 142V- and hGRK4γ WT-transduced cells (culture supernatant: 113.87± 13.07 v.s. 108.73±12.76; cell lysate: 237.3±23.7 v.s. 217±20, n=5, P=NS). The angiotensin converting enzyme inhibitor, captopril (10^{-4} M, Sigma-Aldrich, St. Louis, MO), also had no effect on the AT_1R expression in both cell types (**Supplemental Figure S7**).

As a regulator of AT_1R promoter activity, we measured NF-κB binding to the AT_1R promoter and found it higher in hGRK4γ 142V- than hGRK4γ WT-transduced cells (**Figure 4A**). Blockade of NF-κB with an NF-κB inhibitor, BAY11-7082, inhibited the increase in AT_1R expression in hGRK4γ 142V-transduced cells (**Figure 4B**), indicating that NF-κB was involved in the positive regulation of AT_1R expression by hGRK4γ 142V.

As aforementioned, the decreased AT_1R degradation could be ascribed to the decrease in AT_1R phosphorylation in GRK4γ 142V-transduced cells. An additional study found a co-localization (**Figure 5A**) and co-immunoprecipitation (**Figure 5B**) between GRK4 and AT_1R; the co-immunoprecipitation of GRK4 and AT_1R was less in hGRK4γ 142V- than hGRK4γ WT-transduced cells (**Figure 5B**), which could be a factor in the decreased phosphorylation of AT_1R in hGRK4γ 142V-transduced cells.

3. AT_1R expression and function in hGRK4γ 142V transgenic mice

To further investigate the physiological role of the GRK4-regulated AT_1R expression, we studied AT_1R expression and function in hGRK4γ WT and hGRK4γ 142V transgenic mice. Consistent with previous reports[6, 8, 11, 13], anesthetized hGRK4γ 142V transgenic mice had higher systolic (S), diastolic (D) and mean (M) blood pressures (SBP =123.37 ± 8.19, DBP = 96.37 ± 4.78 mmHg, MBP = 104.54 ± 3.99, n = 11) than anesthetized hGRK4γ WT transgenic mice (SBP = 98.38 ± 5.42, DBP = 83.00 ± 4.54 mmHg, MBP = 88.21 ± 3.63, n = 11, P<0.001). Although GRK4 expression was not different between hGRK4γ WT 142V and hGRK4γ 142V transgenic mice (**Supplemental Figure S5**), AT_1R expression in aorta was higher in hGRK4γ 142V than hGRK4γ WT transgenic mice (**Figure 6A**). We also studied the vasoconstrictor effect of Ang II on the aorta from hGRK4γ 142V and hGRK4γ WT transgenic mice. The vasoconstriction caused by Ang II was greater in hGRK4γ 142V than hGRK4γ WT transgenic mice in the presence or absence of the endothelium. The AT_1R blocker, candesartan (10^{-6}M) blocked the vasoconstrictor effect of Ang II, in both transgenic mice such that there was no longer any difference between the two mouse strains (**Figure 6B**).

Consistent with a previous report[34], the intravenous infusion of Ang II (1 μg/kg/min at rate of 10 μl/h) caused a greater increase in SBP in hGRK4γ 142V than hGRK4γ WT transgenic mice while the intravenous infusion of candesartan (0.139 μg/kg/min at a rate of 10 μl/h) caused a greater decrease in blood pressure in hGRK4γ 142V than hGRK4γ WT transgenic mice(**Supplemental 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.

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There is increasing evidence that GRK4 plays an important role in the pathogenesis of hypertension [6-8, 11, 13, 14, 16, 34]. The GRK4 locus (4p16.3) is linked to and GRK4 gene variants are associated with human essential hypertension [4, 6, 8, 11, 12, 36-38]. In Ghanaians, the two-locus model of angiotensin converting enzyme I/D and GRK4 65L predicts the hypertensive phenotype 70.5% of the time [37]. GRK4 variants, including 65L, 142V, and 486V, by themselves, or interaction with other variants of other genes are associated with hypertension in American Caucasians[39], Australian Caucasians[12], Italians[36] and northern Han Chinese[40]. We have reported that hGRK4γ 142V transgenic mice on 98% C57BL/6J background are hypertensive relative to non-transgenic littermates and hGRK4γ WT transgenic mice [6, 8, 11, 13, 34]. To further investigate the role of GRK4 variants on the hypertension, we generated hGRK4γ 142V and GRK4γ WT transgenic mice on C57BL/6J and SJL/J background. C57BL/6 mice are salt-sensitive while SJL/J mice are salt-resistant [41]. We now report that hGRK4γ 142V 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 [11]. The impaired sodium excretion is mainly due to a dysfunction of the D_1 dopamine receptor [4, 6-8, 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]. While the renal dopaminergic system keeps the blood pressure from increasing following a moderate sodium load [4, 6-8, 11, 14, 42], the renin-angiotensin system, including the AT_1R, is crucial in sodium retention and maintenance of blood pressure, especially under conditions of sodium deficit [5, 8, 15]. Both GRK4 and AT_1R exist in VSMCs, but whether or not GRK4-mediated regulation of blood pressure involves the AT_1R in VSMCs is not known. Our present study found that compared with hGRK4γ WT transgenic mice, hGRK4γ 142V transgenic mice have higher arterial AT_1R expression and Ang II-mediated aortic vasoconstriction. Ang II-mediated increase in intracellular calcium is also increased to a greater extent in hGRK4γ 142V- than hGRK4γ WT-transduced A10 aortic cells The stimulatory effect of hGRK4γ 142V on AT_1R receptor expression and function is physiologically relevant because the intravenous infusion of Ang II increased while the intravenous of infusion of an AT_1R antagonist, candesartan, decreased blood pressure to a greater degree and longer extent in hGRK4γ 142V than 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 AT_1R protein expression are greater in C57BL/6 Jackson than SJL Jackson mice [41]. hGRK4γ 142V transgenic mice on C57BL/6 background are also hypertensive that is caused in part by decreased renal D_1 receptor function [11, 13, 34] and increased renal AT_1R expression [34]. 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 thus, independent of the presence of the salt-resistant phenotype.

As a kinase, GRK4 phosphorylates ligand-unoccupied and -occupied GPCRs as their primary substrates, such as the D_1 dopamine receptor [6-11]. Increased GRK4 activity augments D_1 receptor phosphorylation in kidney [6, 7, 8, 10, 11, 14]. However, our present study found that increased GRK4 activity **decreases** AT_1R phosphorylation, which seems counterintuitive, at first glance. Our experiments uncover a possible mechanism; there is a linkage between GRK4 and AT_1R in VSMCs, and it is interesting to find that the GRK4/ AT_1R linkage is decreased in A10 cells transduced with hGRK4γ 142V, which may therefore cause decrease in AT_1R phosphorylation in the hGRK4γ 142V A10-transduced cells. The decreased phosphorylation of AT_1R in hGRK4γ 142V A10-transduced cells may be involved in the GRK4γ 142V-mediated up-regulation of AT_1R expression, because in present study, we found that a decreased AT_1R degradation accompanies the decreased AT_1R phosphorylation in hGRK4γ 142V-transduced A10 cells. The pathway leading to the

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lower binding of hGRK4γ 142V with AT_1R receptor is not known, which needs to be elucidated in the future.

The regulation hGRK4γ-mediated regulation of AT_1R expression is complicated, as in our present study, we found that in addition to hGRK4γ 142V-mediated decrease in AT_1R degradation, AT_1R transcription is also increased, as evidenced by increased AT_1R mRNA in hGRK4γ 142V-transduced A10 cells. The activity of NF-κB, a regulator of AT_1R promoter activity, is increased, accompanied by an increase in its binding to the AT_1R promoter in hGRK4γ 142V-transduced A10 cells. In the presence of an NF-κB inhibitor, the increase in AT_1R expression in hGRK4γ 142V-transduced A10 cells is abolished, confirming the important role of NF-κB in this process.

Conclusion and Perspectives

Our previous study found that increased renal GRK4 expression causes the attenuated renal D_1 dopamine receptor-mediated natriuresis and diuresis and increased renal AT_1R-mediated sodium excretion that play a role in the pathogenesis of the hypertension in SHRs[14, 16]. The present study reinforces the role of GRK4 in hypertension and shows that a constitutively increased activity of GRK4 increases arterial AT_1R 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What Is New ?

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_1 dopamine receptor and increased renal AT_1R functions. In these studies, we report for the first time that GRK4 is expressed in VSMCs of the aorta and GRK4γ 142V decreased AT_1R degradation, via decreased phosphorylation and increased AT_1R expression, via NF-κB. In A10 cells, expression of GRK4γ 142V augmented the Ang II-mediated increase in intracellular Ca^{2+} levels. In transgenic mice on novel C57Bl/6J and SJL/J background, Ang II-induced vasoconstriction was increased in the aorta from GRK4γ 142V transgenic mice, compared with GRK4γ WT transgenic mice. Finally the hypertension in GRK4γ 142V transgenic mice was related to an increase in Ang II-mediated vasoconstriction.

What Is Relevant ?

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.

Summary

The present study reinforces the role of GRK4 in hypertension and shows that a constitutively increased activity of GRK4 increases arterial AT_1R receptor expression and function, which may be involved in the abnormalities of conduit vessels in essential hypertension.

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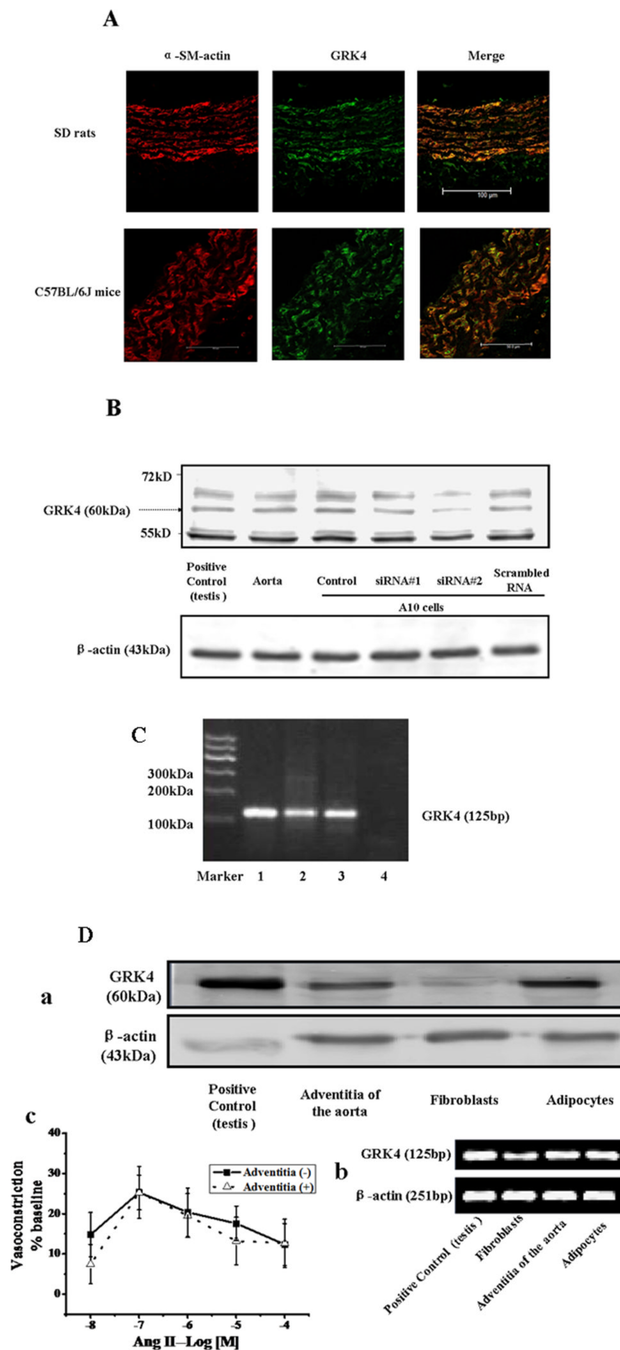

Figure 1. GRK4 expression in aorta

A: Immunofluorescence staining of GRK4 in aorta from SD rats and C57BL/6J mice. The aorta was washed, fixed, and immunostained for GRK4 and α-smooth muscle (SM)-actin, as described in the Methods. Colocalization appears as yellow after merging the images of α-smooth muscle (SM)-actin (red) and GRK4 (green). These studies were repeated at least three times.

B: GRK4 protein expression in A10 cells. Protein (100μg) from A10 cells were subjected to immunoblotting with anti-GRK4 antibody (1:400). The band was attenuated after transfection with the specific GRK4 siRNA into A10 cells (GRK4 siRNA sequence: #1: 5’-AUCUAAAGAGGUGCAUUGAAUUCUUdTdT-3’; #2: 5’-

AAGGACCUCAAUGAAUAUGAAGAUAdTdT-3’) compared with the band of A10 cells without siRNA transfection (scrambled RNA sequence: 5′-TGACGATAAGAACAATAACdTdT-3′). The 54, 60, and 65 kDa bands were found in the aorta and A10 cells, as well as in testis which was used as positive control. These bands are specific GRK4 proteins, as previously published, using the same GRK4 antibody [14]. **C**: GRK4 mRNA expression in aorta from SD rat and A10 cells. GRK4 RT-PCR products from testis (lane 1, positive control), aorta (lane 2) and A10 cells (lane 3) 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 (1μg). No amplification is seen in the absence of RNA (lane 4). **D**: GRK4 expression and function in the adventitia of the aorta. GRK4 expressions were checked in the fibroblasts and adipocytes by immunoblotting (**D-a**) and RT-PCR (**D-b**), samples from testis of SD rats were taken as positive control. Removal of the adventitia did not affect the Ang II (10^{-8}-10^{-}M)-mediated vasoconstriction (**D-c**) (n=4, *P*=NS).

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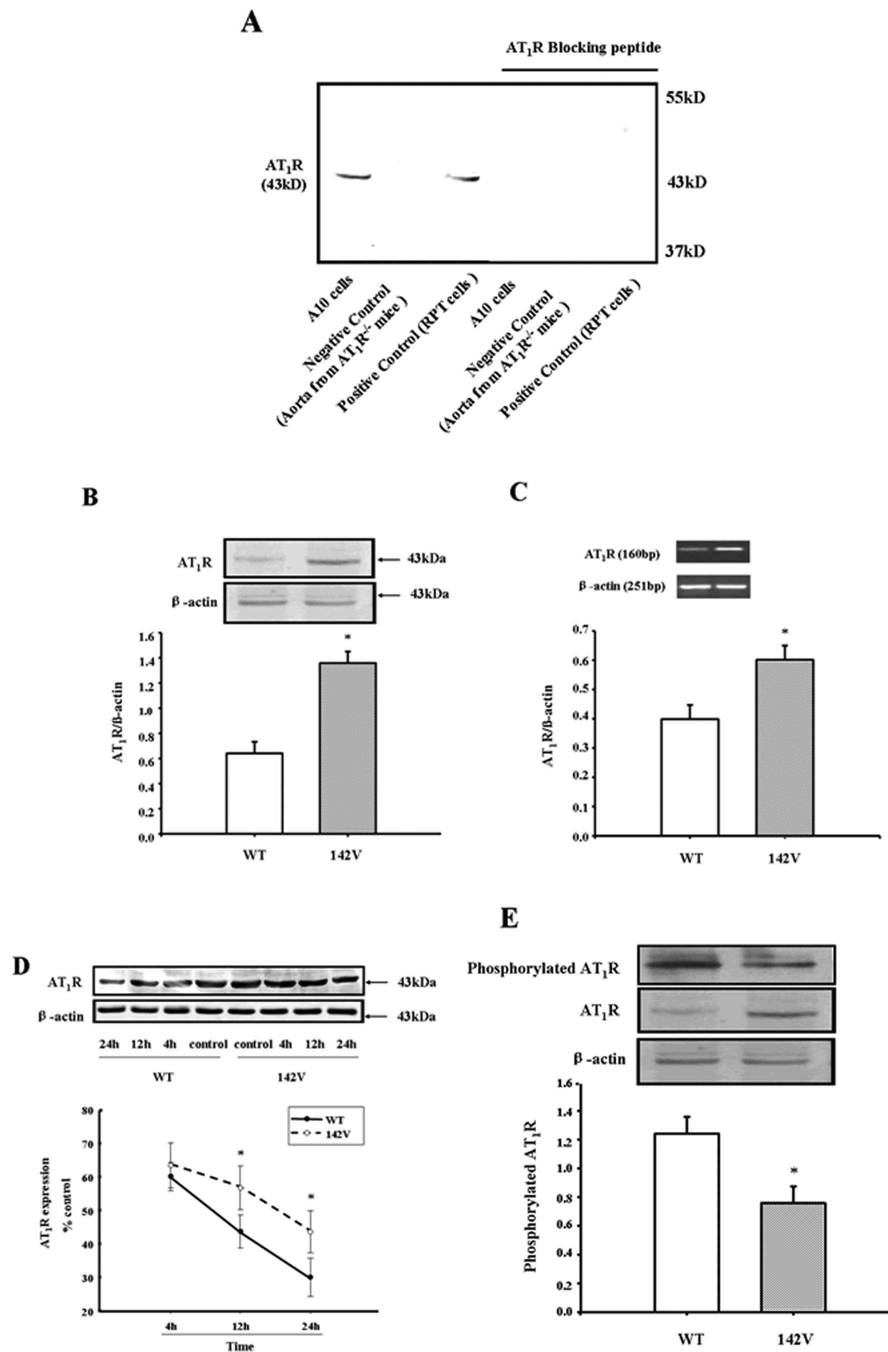

Figure 2. Expression of AT_1R in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells

A: Specificity of AT_1R antibody. Protein (100μg) from A10 cells, renal proximal tubule (RPT) cells from SD rats, and aortae from $AT_1R^{-/-}$ mice were subjected to immunoblotting with anti-AT_1R antibody (1:500) with or without pre-incubation with the AT_1R antibody immunizing peptide (Santa Cruz, 1:10 w/w incubation for 12 hr). These studies were repeated at least three times.

B and C: AT_1R protein (**B** n=7) and mRNA (**C**, n=6) expressions in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells. Results are expressed as the ratio of AT_1R receptor and α-actin (*P<0.05 *vs*. WT)

D: AT_1R protein degradation in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells. The cells were incubated with cycloheximide (10^{-5} M) for the indicated times. Results are expressed as percent change of control in each group ($n = 8$, $^*P < 0.05$ *vs*. WT).

E: AT_1R phosphorylation in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells. The A10 cell lysate protein was immunoprecipitated with anti-phosphoserine antibody and immunoblotted with anti-AT_1R antibody ($n = 3$, $^*P < 0.05$ *vs*. WT).

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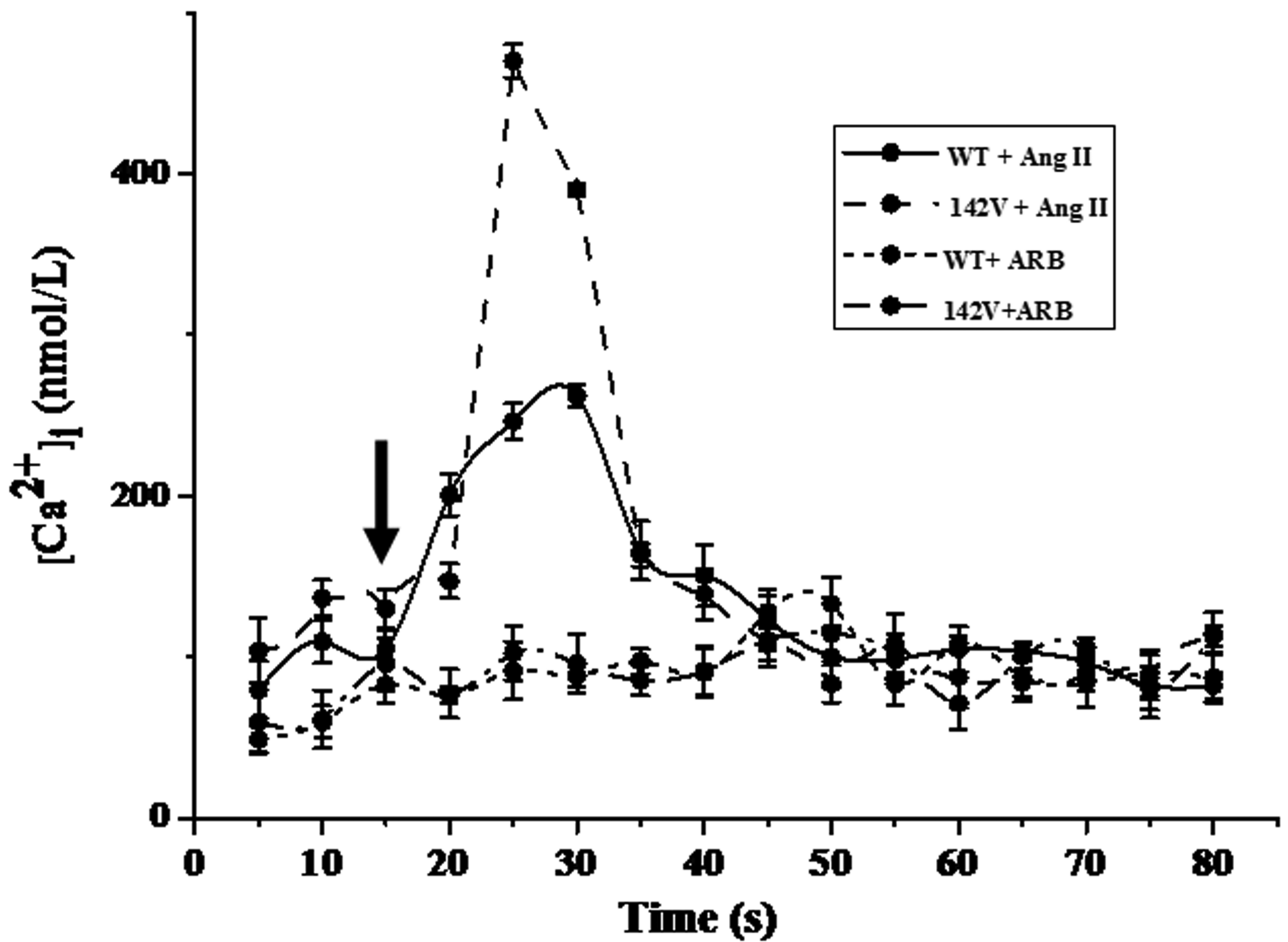

Figure 3.

Intracellular calcium concentration in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells. Representative tracing of the effect of Ang II (10^{-7}M) on intracellular free calcium in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells. Ang II was added 15s after the start of the experiment, shown as the **arrow** in the figure (n=8).

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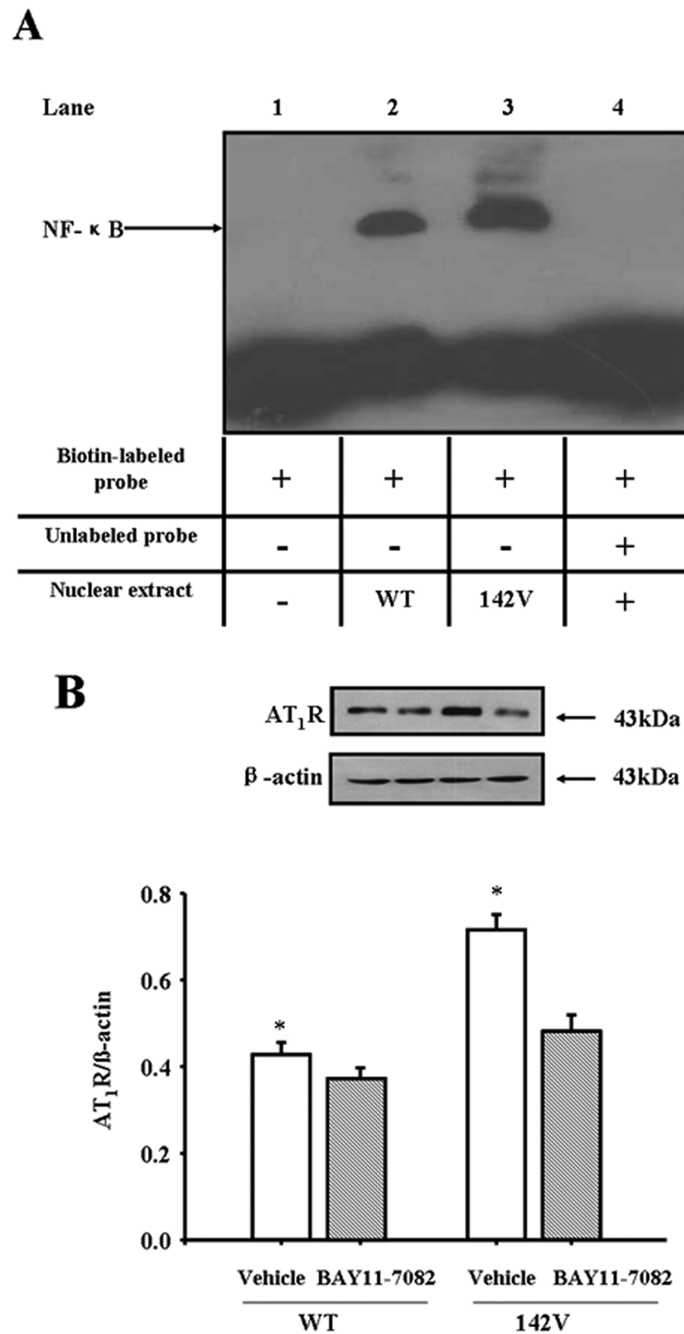

Figure 4. Role of NF-κB on AT_1R expression in GRK4γ WT- and hGRK4γ 142V-transduced A10 cells

A. EMSA of nuclear protein from A10 cells. Binding activity of AT_1R gene promoter (-350 bp and -363 bp), containing an NF-κB site, was examined in nuclear proteins from hGRK4γ WT- (lane 2) and hGRK4γ 142V (lane 3)-transduced A10 cells by EMSA. No nuclear extracts (lane 1) or fifty times unlabeled probe (lane 4) were added to the reaction mixture and served as negative controls. **B**. Effect of NF-κB on GRK4-mediated regulation of AT_1R protein expression in A10 cells. hGRK4γ WT- or hGRK4γ 142V-transduced cells were treated with or without the NF-κB inhibitor BAY11-7082 (20 μM) for 24hr. Results are expressed as the ratio of AT_1R and α-actin (n = 5, *P<0.05 vs. vehicle).

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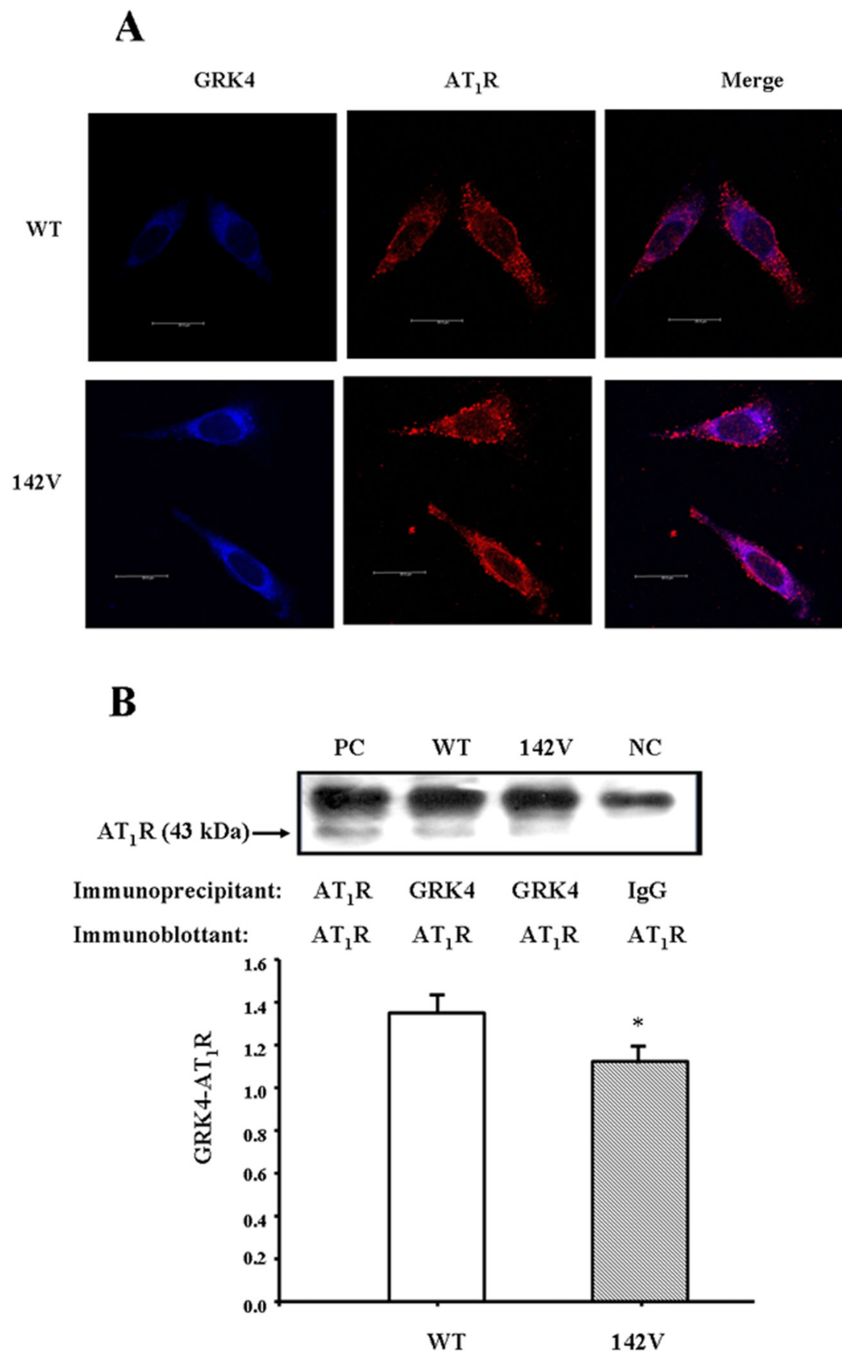

Figure 5. Colocalization and co-immunoprecipitation of GRK4 and AT_1R in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells

A: Colocalization of GRK4 and AT_1R in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells. The cells were washed, fixed, and immunostained for GRK4 and AT_1R, as described in the Methods. Colocalization appears as purple after merging the images of AMCA-tagged GRK4 (blue) and rhodamine-tagged AT_1R (red). **B**: Co-immunoprecipitation of GRK4 and AT_1R in hGRK4γ WT- and hGRK4γ 142V-transduced A10 cells. The cells were immunoprecipitated with GRK4 antibodies and immunoblotted with AT_1R antibodies (*P<0.05 vs. control, n=4, ANOVA, Holm-Sidak test). One immunoblot (43 kDa) is depicted in the inset: (PC = positive control, WT = hGRK4γ WT-transduced A10 cells,

142V = hGRK4γ 142V-transduced A10 cells, NC = negative control. For the positive control, AT_1R antibody was used and for the negative control, IgG was used instead of GRK4 antibody as the immunoprecipitants.

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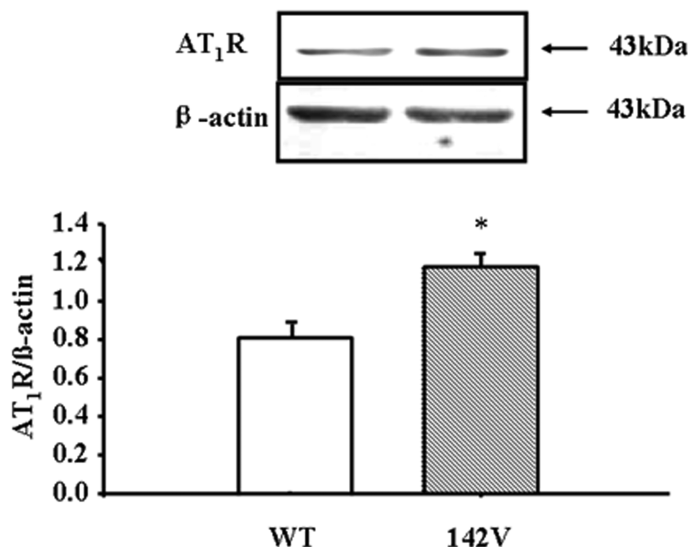

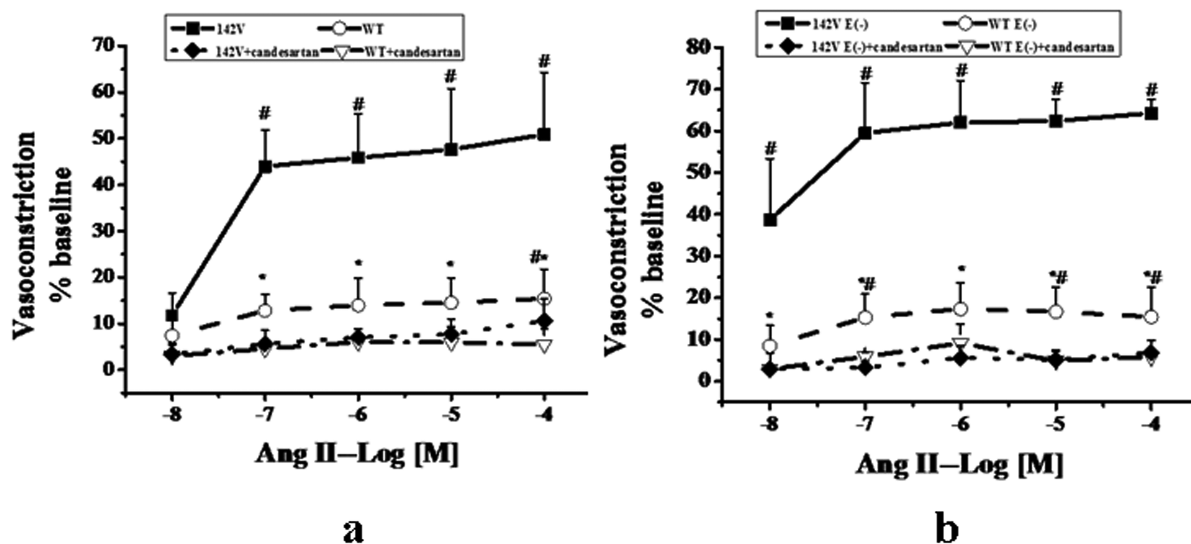

Figure 6. AT_1R expression and function in hGRK4γ 142V transgenic mice

A: AT_1R expression in aortae from hGRK4γ 142V transgenic mice. Aortic homogenates (50μg) from hGRK4γ 142V and hGRK4γ WT transgenic mice were subjected to immunoblotting with anti-AT_1R antibody (1:400). Results are expressed as the ratio of AT_1R to ß-actin densities (n =5, **P*<0.05 vs. control, *t*-test). **B**: Aortic rings with (**B-a**) or without (**B-b**) endothelium (E-) from hGRK4γ WT and hGRK4γ 142V transgenic mice were exposed to varying concentrations of Ang II (10^{-8}-10^{-4}M). To determine the specificity of the Ang II effect on the AT_1R, candesartan (ARB, 10^{-6}M) was added 15 min before the Ang

II treatment. The results are expressed as percent change from baseline. (*$P<0.05$ vs. 142V control, $^{\#}P<0.05$ vs. corresponding group with ARB treatment, n = 12).

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