HHS Public Access

Author manuscript

Hypertension. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

Hypertension. 2016 February ; 67(2): 325–334. doi:10.1161/HYPERTENSIONAHA.115.05962.

HUMAN *GRK4γ*^{142V} VARIANT PROMOTES AT$_1$R-MEDIATED HYPERTENSION VIA RENAL HDAC1 INHIBITION

Zheng Wang[*,1], **Chunyu Zeng**[*,2], **Van Anthony M. Villar**[3], **Shi-You Chen**[4], **Prasad Konkalmatt**[3], **Xiaoyan Wang**[3], **Laureano D. Asico**[3], **John E. Jones**[3], **Yu Yang**[3], **Hironobu Sanada**[5], **Robin A. Felder**[6], **Gilbert M. Eisner**[7], **Matthew R. Weir**[3], **Ines Armando**[3], and **Pedro A. Jose**[3,8,9,**]

[1]Division of Pediatric Nephrology, Department of Pediatrics, Georgetown University of School of Medicine, Washington, DC 20007, USA

[2]Department of Cardiology, Daping Hospital, The Third Military Medical University, and Chongqing Institute of Cardiology, Chongqing, P.R. China

[3]Division of Nephrology, Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA

[4]Department of Physiology & Pharmacology, University of Georgia, Athens, GA 30602, USA

[5]Division of Health Science Research, Fukushima Welfare Federation of Agricultural Cooperatives, Fukushima, Japan

[6]Department of Pathology, The University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA

[7]Department of Medicine, Georgetown University Medical Center, Washington, DC 20007, USA

[8]Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

[9]Department of Medicine, Division of Renal Diseases & Hypertension, and Department of Physiology, The George Washington University School of Medicine & Health Sciences, Washington, DC 20052

Abstract

The influence of a single gene on the etiology of essential hypertension may be difficult to ascertain, unless the gene interacts with other genes that are germane to blood pressure regulation. G protein-coupled receptor kinase type 4 (*GRK4*) is one such gene. We have reported that the expression of its variant *hGRK4γ*^{142V} in mice results in hypertension due to impaired dopamine D$_1$ receptor (D$_1$R). Signaling through D$_1$R and angiotensin II type I receptor (AT$_1$R) reciprocally

[**]To whom correspondence should be addressed: Pedro A. Jose, MD, PhD, The George Washington University School of Medicine & Health Sciences, Ross Hall Suite 738-C, 2300 I St, NW, Washington, DC 20037, pjose@mfa.gwu.edu, Phone: +1-(202)-994-0195, Fax: +1-(202)-994-0926.

[*]These authors contributed equally to this work.

CONFLICT OF INTEREST

Drs. Jose and Felder own Hypogen, Inc., which owns the US Patent (6,660,474B1) for GRK4. Dr. Eisner is a member of the Board of Hypogen, Inc.

modulates renal sodium excretion and blood pressure. Here, we demonstrate the ability of the hGRK4γ^{142V} to increase the expression and activity of the AT_1R. We show that hGRK4γ^{142V} phosphorylates histone deacetylase type 1 and promotes its nuclear export to the cytoplasm, resulting in increased AT_1R expression and greater pressor response to angiotensin II. AT_1R blockade and the deletion of the *Agtr1a* gene normalize the hypertension in *hGRK4*γ^{142V} mice. These findings illustrate the unique role of GRK4 by targeting receptors with opposite physiological activity for the same goal of maintaining blood pressure homeostasis, and thus making the GRK4 a relevant therapeutic target to control blood pressure.

Keywords

Angiotensin II Type 1 receptor (AT_1R); Dopamine D_1 receptor (D_1R); G protein-coupled receptor kinase 4 (*GRK4*); Hypertension; Histone deacetylase (HDAC); Knockout mouse

INTRODUCTION

The genetic basis for essential hypertension has been difficult to decipher. Considering that the intricate control of blood pressure (BP) is governed by a myriad of anatomical, physiological, and biochemical systems, multiple genes are likely to influence an individual's BP and susceptibility to develop hypertension. The impact of any single gene may not overtly manifest since its effects may be reduced and diluted as these are transmitted across many layers of biological control. However, a gene whose product affects the function or expression of several other genes would conceivably have a greater combined effect on BP and thus, would be an attractive target in the study of new approaches to the management of hypertension. The G protein-coupled receptor kinase 4 (GRK4) is one such gene. It plays a critical role in the activity of the peripheral dopaminergic system by phosphorylating and desensitizing the renal dopamine D_1 and D_3 receptors (D_1R and D_3R, respectively) (1,2). These receptors promote natriuresis, to keep BP in the normal range (1,3).

The members of the GRK family are divided into three subfamilies: GRK1 and GRK7 belong to the rhodopsin kinase subfamily; GRK2 and GRK3 belong to the β-adrenergic receptor kinase subfamily; and GRK4, GRK5, and GRK6 belong to the GRK4 subfamily. The expression of GRK4 is limited to only a few organs, such as the brain (4) and kidney (1,3), which are important in the regulation of BP (5–8). The human *GRK4* gene has at least four splice variants (4,9), denoted as GRK4α (longest form), GRK4β (no exon 2), GRK4γ (no exon 15), and GRK4δ (no exons 2 and 15). The GRK4γ isoform desensitizes the D_1R and D_3R (1,2). The *GRK4* gene, whose locus at 4p16.3 is linked to hypertension (10), has several non-synonymous gene variants that are associated with hypertension in several ethnic groups (11–14). These variants increase the serine phosphorylation of the dopamine receptors and uncouple them from their G proteins (1), rendering them dysfunctional. The D_1R/G protein uncoupling in hypertension is kidney-restricted, nephron segment-specific, and receptor-specific, and co-segregates with the hypertension in spontaneously hypertensive rat (SHR) (1). Mice harboring the human *hGRK4*γ^{142V} variant (missense A142V; rs1024323) transgene develop hypertension (1), demonstrating for the first time the

causal role of a gene variant in hypertension. Variants of four other genes have so far been demonstrated to cause hypertension when heterologously expressed in mice, namely, *AGT* (15) that encodes angiotensinogen, *AGTR1* (16) that encodes the angiotensin II (AngII) type 1 receptor (AT_1R) , *CYP11B2* that encodes aldosterone synthase (17), and *UMOD* (18) that encodes uromodulin.

The hypertension in *hGRK4*γ^{142V} transgenic mice has been ascribed to the impairment of D_1R (1). However, studies in SHRs, normotensive Wistar Kyoto (WKY) rats, old rats, and salt-sensitive mice suggest that renal GRK4 and AT_1R interaction may be crucial to the overall regulation of sodium balance and BP (19–21). The AT_1R is activated by AngII to promote vasoconstriction, anti-natriuresis, and sympathetic nervous activation, resulting in increased BP (5–7). The current study tested the hypothesis that the hypertension in *hGRK4*γ^{142V} transgenic mice is caused, in part, by increased AT_1R expression and activity, and highlights the critical role of GRK4 as an upstream regulator of both D_1R and AT_1R in the control of renal sodium excretion and BP.

MATERIALS & METHODS

Details are in the “Online Supplement”.

Cell culture and transfection

HEK-293 cells (ATCC, CRL-1573) were transfected with vectors expressing *hGRK4*γ^{WT} or *hGRK4*γ^{142V} using *Trans*IT-293 under zeocin selection. *Mycoplasma*-negative human renal proximal tubule cells (hRPTCs) from a normotensive Caucasian male (1) were grown in 12-well plates for transient transfection using Fugene 6.

Nuclear expression of hGRK4γ

HEK-293 cells, stably transfected with *hGRK4*γ^{WT} or *hGRK4*γ^{142V}, and hRPTCs were grown on BioCoat™ coverslips (BD Bioscience), immunostained for GRK4 and AT_1R, and counterstained with DAPI to visualize the nuclei. Nuclear and cytosolic fractions from hRPTCs and HEK-293 cells stably transfected with *hGRK4*γ^{WT} or *hGRK4*γ^{142V} were prepared for immunoblotting. Histone deacetylase (HDAC) activity was fluorometrically measured.

Human AT_1R gene (*AGTR1*) promoter analysis

Human *AGTR1* promoter DNA fragment from −1941 to +281 relative to the transcription start site was cloned into pGL3 Luciferase reporter vector and co-transfected with pcDNA, *hGRK4*γ^{WT}, *hGRK4*γ^{142V}, or *hGRK2* and a constant amount of plasmid harboring Renilla luciferase into hRPTCs. The cells were incubated for 24 hr followed by luciferase assay.

Generation of *hGRK4*γ^{WT} and *hGRK4*γ^{142V} transgenic mice

We generated *hGRK4*γ^{WT} and *hGRK4*γ^{142V} transgenic mice to determine causality between *GRK4* variants and BP (1). GRK4γ was chosen for this study because it regulates a limited number of GPCRs (4), including the D_1R and D_3R which are important regulators of blood pressure (1–3,8,19,20). Age- and sex-matched 3–8 month-old (N5-N6) mice on a ≈98%

C57BL/6 and ≈2% SJL background were studied. These mice do not have *Grk4* mutations and their blood pressures are normal when fed a normal salt diet. However, high salt diet increased the blood pressure and renal GRK4 protein expression in the salt-sensitive C57BL/6 but not in the salt-resistant SJL/J mice (21).

Generation of *hGRK4^{142V}/Agtr1a $^{+/-}$* and *GRK4^{142V}/Agtr1a$^{-/-}$* mice

The role of *Agtr1a* in the hypertension of *GRK4γ^{142V}* mice (>98% C57BL/6 background) was studied by cross-breeding with *Agtr1a$^{-/-}$* mice (75% C57BL/6 and 25% 129S/SvEv background).

Generation of *hGRK4^{142V}/GRK4 $^{-/-}$* mice

hGRK4^{142V}/GRK4$^{-/-}$ mice were generated by breeding *hGRK4^{142V}/GRK4$^{+/-}$* mice with *hGRK4^{142V}/GRK4$^{+/-}$* mice.

Acute renal-specific down-regulation of *Hdac1or Hdac2*

Renal *Hdac1* was silenced through a renal subcapsular infusion of *Hdac1*-specific siRNA via an osmotic minipump (22). The osmotic minipumps (100 μl; flow rate: 0.5 μl/hr for 7 days) were filled with pre-validated *Hdac1*- or *Hdac2*-specific siRNA (delivery rate of 3μg/day) or non-silencing siRNA as control. The siRNAs were dissolved in an *in vivo* transfection reagent under sterile conditions.

BP measurement in transgenic mice

BP of mice fed a normal (0.9% NaCl) or high (6% NaCl) salt diet was measured directly from the aorta via the femoral artery under pentobarbital anesthesia, or by telemetry via the carotid artery in conscious, undisturbed mice starting at 7 days after the surgical implantation of the transmitters (21).

AngII and candesartan treatment

The mice were anesthetized with isoflurane for the subcutaneous implantation of osmotic minipumps to administer AngII (1 μg/kg/min at the rate of 1 μl/hr) or candesartan (0.139 μg/kg/min at the rate of 0.5 μl/hr/4 days). BP was measured in anesthetized (1) and conscious mice (21).

RT-PCR and real-time qPCR

The transgene copy number was calculated using the absolute quantitative method.

Immunoblotting

Renal sodium transporters, pump, and channels: kidneys were prepared for immunoblotting using rabbit polyclonal antibodies against NHE3, NaPi2, NKCC2, NCC, ENaC subunits (gifts from Dr. Mark A. Knepper, ESBL, NHLBI, NIH), actin (housekeeping protein), and mouse monoclonal antibody against Na^+, K^+-ATPase α subunit.

Renal AT_1R: Mouse kidney proteins were probed with antibodies against AT_1R, β-actin, and GRK4.

Generation of anti-GRK4 antibody

Rabbit anti-mouse GRK4 (peptide sequence: KDLNENEDDLSSLEKYK) antibody was generated. The specificity of the antibody was validated by blot.

Urine sodium and creatinine assays

Mouse urine samples were collected in metabolic cages for 24 hr under basal conditions. The urine sodium was determined using the Beckman Synchron EL-ISE electrolytes system and creatinine was measured by using QuantiChrom™ Creatinine Assay Kit.

Angiotensinogen, ACE, and renin assays

Plasma total angiotensinogen was measured using an EIA kit. Serum ACE activity was also measured with a commercially available kit. Plasma (treated with EDTA and PMSF) renin activity was measured as the rate of AngI generation in the presence of excess exogenous substrate. AngI was quantified by RIA using a clinical human renin kit. All assays were performed by the Hypertension Core Laboratory, Wake Forest University School of Medicine.

Study approval

All animals used in this study were bred and maintained at the animal facility at Georgetown University and University of Maryland School of Medicine. The studies were conducted in accordance with NIH guidelines for the care and use of laboratory animals in research, and approved by Georgetown University and the University of Maryland School of Medicine Institutional Animal Care and Use Committees (IACUC).

Statistics

The data are expressed as mean ± s.e.m. Significant differences between and among groups were determined by one-way ANOVA and Scheffe's or Holm-Sidak test for groups >2 or Student's *t*-test for groups=2. $P<0.05$ was considered significant.

RESULTS

hGRK4γ^{142V} is more abundant than the wild-type isoform in the nucleus

We initially determined the renal expression profiles of AT_1R in *hGRK4γ^{142V}* transgenic mice and those expressing the wild-type human GRK4 (*hGRK4γ^{WT}*). The renal AT_1R transcript and protein levels were greater in male and female *GRK4γ^{142V}* than in *GRK4γ^{WT}* transgenic mice (Figure 1A), suggesting a regulatory role for *hGRK4γ^{142V}* on AT_1R expression. To elucidate the mechanism for the up-regulation of AT_1R, we determined the subcellular localization of heterologous hGRK4γ^{142V} and hGRK4γ^{WT} in HEK-293 cells, which have functional endogenous AT_1R but minimal GRK4 expression. Both forms of GRK4 were distributed in the plasma membrane, cytosol, and the nucleus (Figure 1B), similar to the distribution of endogenous GRK4 in hRPTCs (1) (Figure 1B). We confirmed the nuclear localization of GRK4γ by immunoblotting nuclear and cytoplasmic fractions and found that the nuclear/cytoplasmic ratio of hGRK4γ was significantly higher in cells expressing *hGRK4γ^{142V}* than for *hGRK4γ^{WT}* (Figure 1C), similar to the distribution of

endogenous GRK4 in several hRPTC lines (Figure 1D). GRK4 contains a putative nuclear localization signal (23). GRK5, which has a well-characterized nuclear localization signal (24,25), negatively modifies the transcription of several $G_{\alpha q}$-regulated genes and acts as a kinase for HDAC5 (25,26). HDACs remove acetyl groups from lysine residues of histones, allowing the histones to bind tightly with DNA, preventing transcription. However, activation via serine phosphorylation of Class II HDACs results in HDAC transport into the cytoplasm (25), preventing action on the nuclear histones, leading to a relaxed conformation of the nucleosome, allowing gene expression to occur.

hGRK4γ^{142V} promotes HDAC1 egress from the nucleus and up-regulates AT_1R expression

The importance of HDACs in hypertension has been reported and their effects may be organ-specific. In the kidney, the inhibition of total HDAC activity increases the transcription of RAAS genes, including *Agtr1*, while AngII increases HDAC1 expression (27). In extra-renal tissues, systemic HDAC inhibition decreases the hypertensive response by decreasing AT_1R expression in the heart of SHRs (28), while the class III HDAC sirtuin 1 down-regulates AT_1R expression and AngII activates HDAC5 in vascular smooth muscle cells (29,30). We found in hRPTCs that endogenous GRK4 co-immunoprecipitated with endogenous HDAC1 (Figure 2A). The expression of hGRK4^{142V} in hRPTCs increased HDAC1 phosphorylation (Figure 2B), decreased nuclear HDAC1 activity (Figure 2C), and increased AT_1R expression (Figure 2D), relative to those in cells expressing hGRK4^{WT}. Thus hGRK4^{142V} promotes HDAC1 activation and nuclear export to the cytoplasm, resulting in increased AT_1R expression.

We next evaluated the effect of hGRK4γ^{142V} and hGRK4γ^{WT} on the activity of the *AGTR1* promoter in hRPTCs. The *AGTR1* promoter spans ~2.2 kb from –1941 to +281 nucleotides relative to the transcriptional start site and contains most of the regulatory elements that control the expression of the AT_1R (31). The CpG island predictor tool (http://www.urogene.org/methprimer) showed that the human *AGTR1* promoter contains a 461-bp CpG island at −304 to +156 nucleotide position that can be acted upon by the HDACs. We found that hGRK4γ^{WT}, but not GRK2, inhibited the activity of the *AGTR1* promoter, and thus gene expression, while hGRK4γ^{142V} markedly enhanced its activity (Figure 2E).

Depletion of renal HDAC1 increases AT_1R expression and BP in mice

We next infused *Hdac1*-specific siRNA into the kidney of C57Bl/6J mice for 7 days; the *hGRK4γ^{142V}* mice are on a C57Bl/6J genetic background. *Hdac2*-specific and non-silencing siRNA were used as controls. Depletion of renal HDAC1 increased renal *Agtr1* expression (Figure 3A) and elevated systolic BP (Figure 3B), which was reduced by AT_1R blockade with candesartan (Figure 3C) in another set of mice. Depletion of renal HDAC2 did not alter the AT_1R expression (Figure 3A) but decreased the systolic BP (Figure 3B). The reason for the decreased BP with renal HDAC2 depletion is unclear, although it is not due to increased renal HDAC1 since HDAC1 expression in total kidney homogenates was unchanged with HDAC2 depletion (Figure 3D); HDAC2 expression was not altered by renal HDAC1 depletion (Figure 3E).

hGRK4γ^{142V} transgenic mice have decreased renal HDAC1 and hypertension

Mice harboring the *hGRK4γ*^{142V} are viable, healthy, and indistinguishable from control mice (1). To eliminate the confounding effects of the endogenous mouse *Grk4* gene, we crossed the *hGRK4γ*^{142V} with *Grk4*$^{-/-}$ mice and studied the mice that express the human transgene but not the endogenous mouse gene (*hGRK4γ*^{142V}/*Grk4*$^{-/-}$). *hGRK4γ*^{142V} mice, with or without the endogenous *Grk4* gene, had systolic and diastolic BPs that were higher than in control mice (Figure 4A), in agreement with previous studies (1). We also found that *Grk4*$^{-/-}$ mice had low basal BP, indicating that in the C57BL/6J mouse, which has increased renal GRK4 expression on high salt intake (21), GRK4 constrains the D_1R function. Conscious *hGRK4γ*^{142V} mice had elevated BP throughout most of the day and were hypertensive at night when they were awake and active, in contrast to non-transgenic and *hGRK4γ*^{WT} mice that had normal BP (Figure 4B). The increased BP of *hGRK4γ*^{142V} mice was independent of age and was not related to *GRK4* transgene copy number (Figure 4C), renal *GRK4* transgene mRNA level (Figure 4D), or GRK4 protein expression, that was not affected by sex (Figure 4E). Salt loading did not increase further the high BP of *hGRK4γ*^{142V} mice (Figure 4F), although it increased the BP of non-transgenic littermates, which were on a salt-sensitive C57Bl/6J genetic background (21). The increased BP correlated with the reduction of HDAC1 in whole kidney homogenates and nuclear fractions, but not in cytosolic fractions, in *hGRK4γ*^{142V} mice compared with *hGRK4γ*^{WT} mice (Figure 4G), corroborating the regulatory role of hGRK4γ^{142V} on HDAC1 expression, activation, and subcellular localization (Figures 2 and 3).

hGRK4γ^{142V} transgenic mice have lower plasma renin activity

We also explored the interaction between GRK4 and the RAAS by evaluating the expression and activity of RAAS components. *hGRK4γ*^{142V} mice had lower plasma renin activity than *hGRK4γ*^{WT} mice, although there were no differences in plasma angiotensinogen concentrations and serum angiotensin converting enzyme (ACE) activities (Supplementary Figure 1). The lower plasma renin activity is consistent with the association of *hGRK4γ*^{142V} and low plasma renin activity in a hypertensive Japanese population with *GRK4* gene variants (13). Stimulation of the mouse D_1R, which is expressed in the juxtaglomerular (JG) cells, increases renin secretion (32), when cyclooxygenase-2 is suppressed (33), while stimulation of the AT_1R, which is also expressed in JG cells (34), down-regulates renin synthesis and secretion (35). The expression of hGRK4γ^{142V} impairs the D_1R activity and heightens AT_1R activity, which together result in reduced renin secretion. Urine aldosterone levels were not different between *hGRK4γ*^{142V} and *hGRK4γ*^{WT} transgenic mice (16.06±7.42 ng/mg creatinine *vs*. 17.53±9.06, *hGRK4γ*^{142V}; n=4/group).

AT_1R blockade or loss prevents the hGRK4γ^{142V}-mediated hypertension in mice

The acute subcutaneous infusion of AngII increased daytime BP to a greater extent in conscious *hGRK4γ*^{142V} than in *hGRK4γ*^{WT} mice (Figure 5A), conceivably because *hGRK4γ*^{142V} mice had more AT_1R (Figure 1A). A chronic subcutaneous infusion of the AT_1R blocker candesartan decreased daytime BP to a greater extent in *hGRK4γ*^{142V} than *hGRK4γ*^{WT} mice, such that their actual BPs became the same (Figures 5B and 5C), indicating an unfettered AT_1R activity in *hGRK4γ*^{142V} mice and tonic AT_1R activity in

hGRK4γ^{WT} mice. To corroborate the role of AT_1R in the hypertension observed in *hGRK4γ*^{142V} mice, we crossed these mice with *Agtr1a*$^{-/-}$ mice and observed that the loss of one of the two *Agtr1a* alleles was sufficient to prevent the "hypertensinogenic" effect of *hGRK4γ*^{142V} (Figure 5D), regardless of sex (Figure 5E).

Chronic hypertension develops when a higher BP is needed to excrete the same amount of sodium as when the BP is normal, *i.e.*, there is a shift of the pressure-natriuresis curve to the right (7), which was observed in the hypertensive *hGRK4γ*^{142V} mice (Figure 5F). The shift in the pressure-natriuresis plot to the right may be due to: (a) increased renal AT_1R expression and function (*current study*); (b) decreased renal dopamine receptor expression and/or function (1,3,8,20); and (c) ultimately, to increased sodium transport that may be related to increased ion transporter expression, i.e., NKCC2 (Figure 5G) or activity.

DISCUSSION

The intricate regulation of renal sodium transport and BP is mainly governed by the combined effects of two contrasting systems, i.e., the anti-hypertensinogenic (e.g., peripheral dopamine system) and pro-hypertensinogenic systems (e.g., RAAS and sympathetic nervous system). This "push and pull" dynamic is best exemplified by the interaction between the D_1R and AT_1R. These receptors, along with Na^+,K^+-ATPase, comprise a multi-protein complex where one receptor exerts antagonizes the other (36,37). The D_1R possesses a region at the C-terminal tail that can directly interact with both the AT_1R and Na^+,K^+-ATPase (36). This may allow an acute, short-term D_1R agonism to cause the rapid partial internalization of AT_1R and complete abrogation of AT_1R signaling (36). Similarly, short-term treatment with AngII diminishes D_1R at the plasma membrane in the WKY, but not SHR, although chronic treatment results in increased D_1R expression (37), possibly as compensation. AngII also decreases dopamine uptake and dopamine synthesis in the renal cortex (38). Simultaneous activation of D_1R and AT_1R can increase or decrease Na^+,K^+-ATPase activity that is dependent on the intracellular sodium concentration, an increase of which favors D_1R action (39). The reciprocal antagonism between D_1R and AT_1R is also carried out by exerting opposite effects on a common intracellular signaling pathway (i.e., adenylyl cyclase/cAMP), and on the same targeted effector protein (e.g., Na^+,K^+-ATPase) (37,40).

We, now, identify and demonstrate the unique role of GRK4 as a shared modulator for D_1R and AT_1R expression and activity in the long-term (Figure 6). The expression of *hGRK4γ*^{142V} has a two-pronged effect. First, by acting in a conventional GRK fashion, it impairs D_1R (1,19,21,41) and D_3R function (unpublished data), leading to the loss of natriuretic response during moderate sodium excess (1). Second, by acting in a non-GRK fashion, it phosphorylates HDAC1, leading to increased AT_1R expression and activity, and anti-natriuresis. These result in high BP, thus highlighting the crucial role of the GRK4 variant in hypertension. Indeed, alterations in both D_1R (diminished activity) and AT_1R (enhanced activity) have been reported as concurrent requirements for hypertension (19,20,41). *hGRK4γ*^{142V} increased AT_1R in rat aorta-derived A10 vascular smooth muscle cells and aorta of *hGRK4γ*^{142V} transgenic mice, causing a greater AngII-mediated vasoconstriction (42). This demonstrates that the regulation of AT_1R expression by

hGRK4γ^{142V} extends to extra-renal tissues and may play an important contributory role in hypertension.

The apparent deleterious effect of HDAC1 inhibition by GRK4γ^{142V} may seem to contradict the beneficial effects of systemic HDAC inhibitors on inflammation, asthma (43), pulmonary circulation (44), oncogenesis (45), kidney disease (46), myocardial hypertrophy (47,48), and BP control (28,49). However, Class 1 HDAC inhibition may have deleterious effects on cardiac function (50). These contrasting reports could be related to the fact that the HDAC inhibitors used were HDAC class-specific and not subtype-specific. Our current studies show that renal-selective down-regulation of HDAC1 increased BP while renal-selective down-regulation of HDAC2 decreased BP. A recent review indicated the lack of information on the regulators of Class I HDACs (48). The current studies show that GRK4 selectively regulates HDAC1 but not HDAC2.

Our findings highlight the relevance of GRK4 in the etiology of hypertension. The *GRK4* gene is one of the few genes that fulfill the criteria (51,52) for ascribing a gene as causal of a complex disorder, such as hypertension. These include supporting data from linkage studies in several ethnic populations, comprehensive sequence analyses, *in vitro* studies using pertinent cell lines, and definitive evidence involving transgenics and gene knockout models. GWAS, which have identified only about 2% of the genetic factors believed to influence BP (53), failed to associate the *GRK4* variants with hypertension. One reason for the non-association may be the use of stringent correction and requirement for independent replication, resulting in higher type 2 error rates (54). Another reason may be the failure to include epistasis and epigenetics in the analyses (55,56). The importance of gene-gene interactions and epigenetic variations is supported by the association of the *GRK4* variants with variants of other genes that are implicated in the etiology of hypertension (11–14), and by the current study that shows the effect of the *GRK4*^{142V} on HDAC1 activation, and thus on nucleosome conformation and gene expression. Moreover, some of the GWAS arrays used in various analyses do not include all of the 3 GRK4 variants, e.g., Affymetrix chips do not have *GRK4*^{142V}.

Our studies illustrate the interplay between genetic and epigenetic mechanisms that promote the development of hypertension in rodents. Indeed, *GRK4*, by its ability to regulate several genes (e.g., *DRD1*, *DRD3*, and *AGTR1*) involved in renal function and BP control, may be a key to understanding the apparent “polygenic” nature of essential hypertension.

PERSPECTIVES

The pleiotropic effects of the *hGRK4γ*^{142V} that perturb D_1R expression/function and, at the same time, augment AT_1R expression/function demonstrate how one gene may affect other physiologically relevant genes and hence, would have a greater influence on BP and be an attractive target, i.e., pharmacogenetics. Considering that GRK4 affects at least three important regulators of renal sodium excretion and BP, i.e., D_1R, D_3R, and AT_1R, and these receptors, in turn, affect other receptors and proteins that modulate BP homeostasis, the utility of GRK4 and its variants in predicting which drug combination is most effective in managing the disorder is considerable. For example, hypertensive carriers of *hGRK4*^{142V}

had a greater decrease in systolic BP in response to angiotensin receptor blockers than non-carrier hypertensive patients (57). More importantly, this will help in identifying which individuals are at a greater risk to develop hypertension so that timely preventive measures can be instituted. Early identification includes genetic testing for individuals who are pre-hypertensives and the screening of unaffected family members of hypertensive patients. Indeed, our data can easily translate into clinical medicine by providing a framework to predict clinical phenotype, effective drug response, and individualize therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

The *Agtr1a*$^{-/-}$ mice were generously supplied by Thomas M. Coffman, MD, Duke University and Durham VA Medical Centers, and Amy Mangrum, MD, The University of Virginia Health Sciences Center. The *Grk4*$^{-/-}$ mice were generously supplied by Richard T. Premont, PhD, and Robert J. Lefkowitz, MD, Duke University.

SOURCES OF FUNDING:

The work was funded by grants from the US National Institutes of Health, P01HL074940, P01HL068686, R01HL092196, R37HL023081, R01DK039308, and DK090918.

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Highlights

1. **What is new?**
   - Nuclear GRK4 and its variant *GRK4*^{142V}activate HDAC1, but not HDAC2, promote its nuclear export, and allow the transcription of *AGTR1*.
   - HDAC activity and AT_1R expression are increased in hRPTCs carrying *GRK4*^{142V}.
   - Human *GRK4*^{142V} increases BP in mice lacking the endogenous mouse *Grk4* while absence of the endogenous *Grk4* in mice decreases BP.
   - We demonstrated the utility in crossing two genetically modified mouse strains, i.e., *hGRK4*^{142V} transgenic mice and *Agtr1a* $^{-/-}$ mice, to underscore the functional relevance of AT_1R in hypertension.
2. **What is relevant?**
   - *GRK4* is one of only five genes that promote hypertension when its gene variant is heterologously expressed in mice.
   - The presence of *hGRK4*^{142V}, in conjunction with other GRK4 variants and those of other relevant genes, can be used to predict effective antihypertensive drug response and help in the early identification of individuals who are at risk of developing the disease.
3. **Summary**
   - *hGRK4*^{142V} is more abundant than its wild-type version in the nucleus where it activates and phosphorylates HDAC1 but not HDAC2 to promote its egress to the cytoplasm, which in turn, allows the transcription of *AGTR1*.
   - *hGRK4*^{142V} transgenic mice have increased renal AT_1R expression and lower plasma renin activity, and exhibit impaired pressure-natriuresis.
   - Pharmacological blockade or genetic ablation of the *Agtr1a* gene normalizes the high BP of *hGRK4*^{142V} transgenic mice.

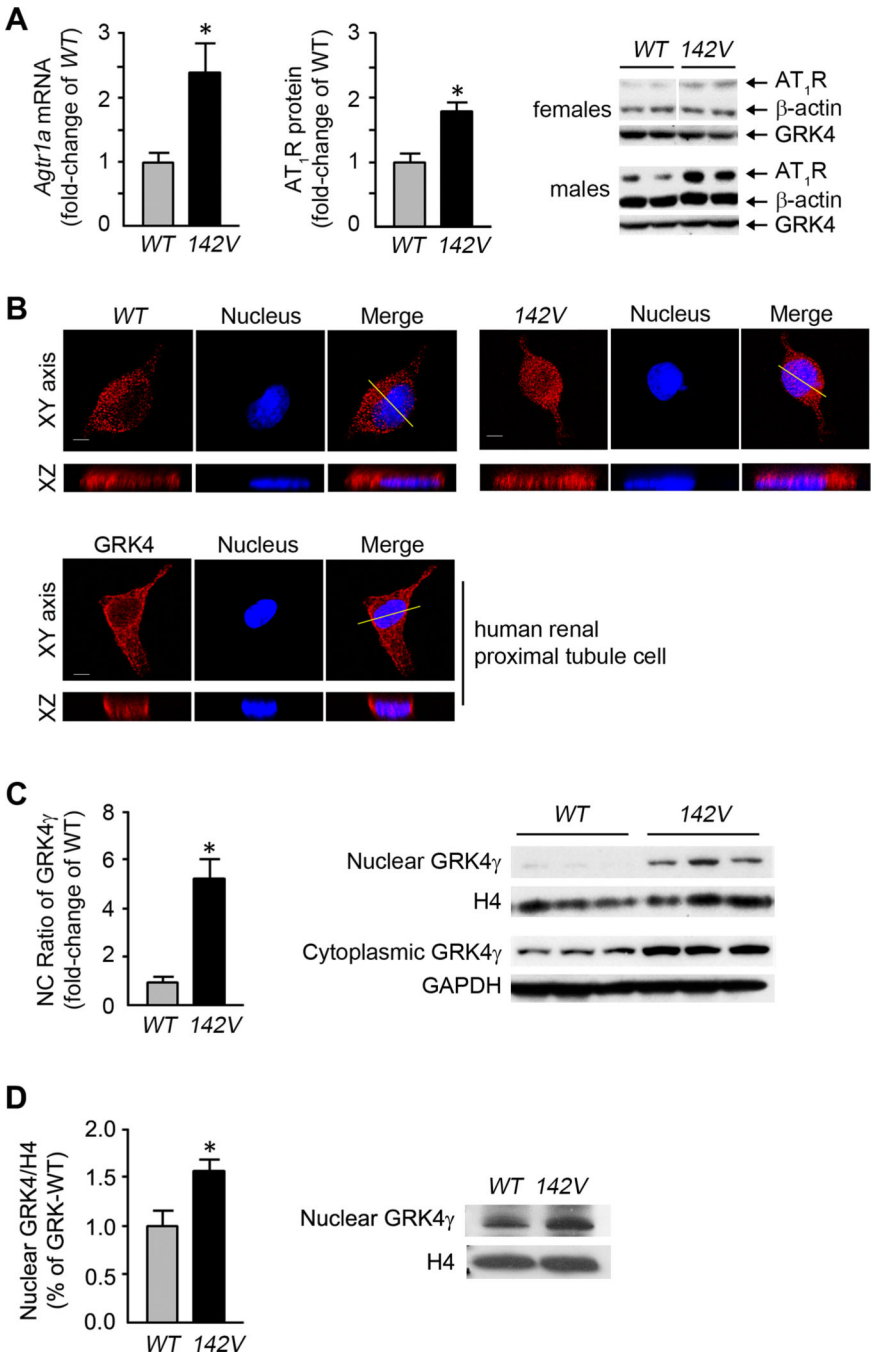

Figure 1. Mouse renal AT_1R expression and hGRK4 subcellular distribution

(**A**) Renal *Agtr1a* mRNA and protein expressions normalized to β-actin and expressed as fold-change from $hGRK4\gamma^{WT}$ were greater in $hGRK4\gamma^{142V}$ (*142V*) than $hGRK4\gamma^{WT}$(*WT*) transgenic mice. Sample immunoblots are shown on the right. Data are expressed as mean ± s.e.m. **P*<0.05, *t*-test, *n*=5–6/group. (**B**) Subcellular localization of heterologous $hGRK4\gamma^{WT}$(*WT*) and $hGRK4\gamma^{142V}$(*142V*) in HEK-293 cells and endogenous GRK4 in hRPTCs. The cells were immunostained for GRK4 and the nuclei were stained using DAPI and visualized at the XYZ axes via confocal microscope. Scale bar = 5 μm, magnification

600X. (**C**) Nuclear/Cytoplasmic (NC) ratio of GRK4γ in HEK-293 cells expressing *hGRK4γ*^{WT}(*WT*) or *hGRK4γ*^{142V}(*142V*). Histone H4 (H4) and GAPDH were used as loading controls for nuclear and cytosolic preparations, respectively. (**D**) Nuclear distribution of endogenous hGRK4γ^{WT} (*WT*) and hGRK4^{142V} (*142V*) in several hRPTC lines. Sample immunoblots are shown on the right. Data are expressed as mean ± s.e.m. **P*<0.05, Student's *t*-test, *n*=5–6/group.

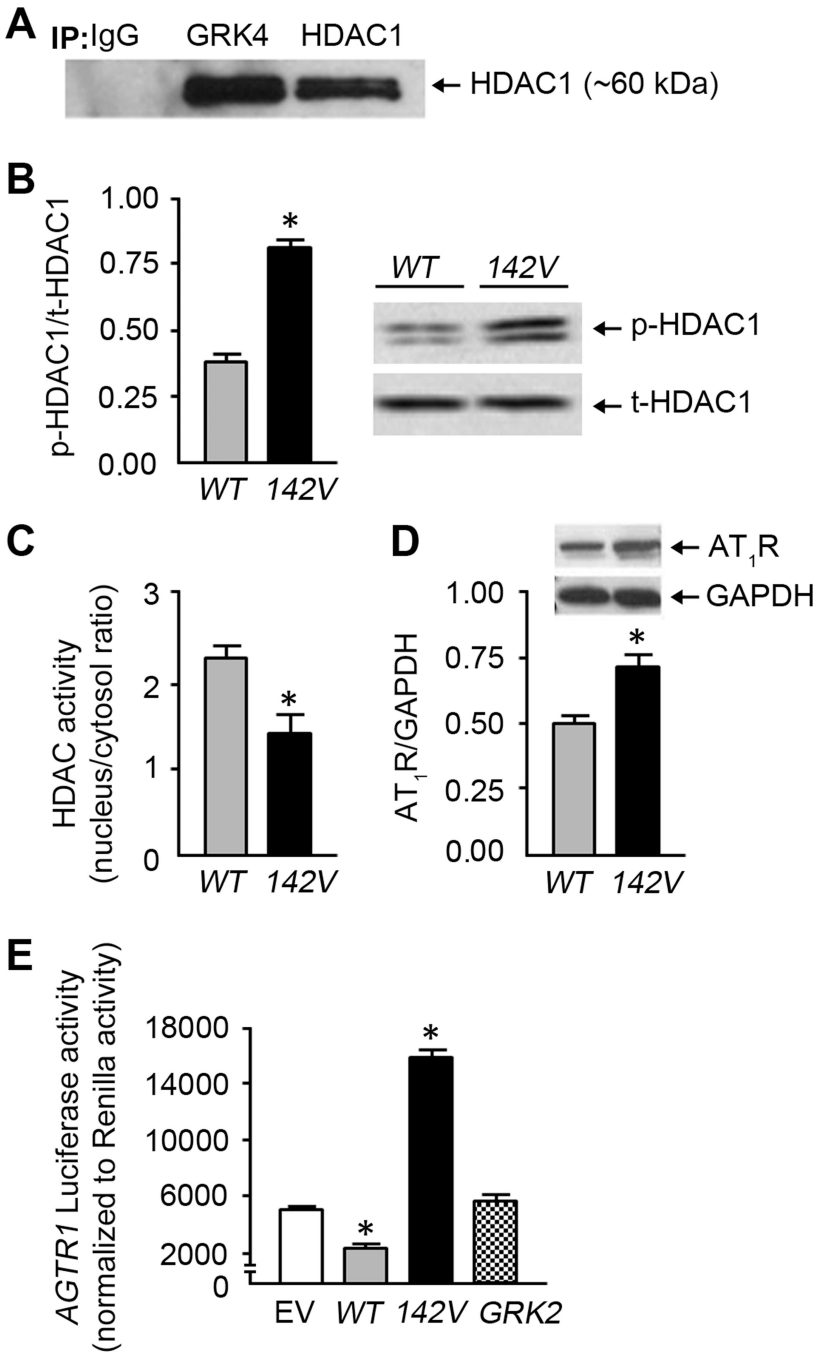

Figure 2. Interaction between GRK4 and HDAC1

(**A**) Co-immunoprecipitation between endogenous GRK4 and HDAC1 in nuclear extracts from hRPTCs. Normal rabbit IgG was used as negative control. Anti-HDAC1 as immunoprecipitant was used as positive control. An immunoblot of the immunoprecipitated HDAC1 is shown. *n*=3 (**B**) Basal HDAC1 phosphorylation in hRPTCs endogenously expressing $hGRK4\gamma^{WT}$(*WT*) or $hGRK4\gamma^{142V}$(*142V*). Nuclear extracts were immunoblotted for phosphorylated (p-HDAC1) and total (t-HDAC1) HDAC1; a sample immunoblot is shown on the right. Data are expressed as mean ± s.e.m. **P*<0.05, *t*-test, *n*=3/group. (**C**)

Total HDAC activity in nuclear and cytosolic fractions of hRPTCs endogenously expressing *hGRK4*γ^{WT}(*WT*) or *hGRK4*γ^{142V}(*142V*). Data are expressed as mean ± s.e.m. **P*<0.05, *t*-test, *n*=3/group. (**D**) AT_1R expression in hRPTCs endogenously expressing *hGRK4*γ^{WT}(*WT*) or *hGRK4*γ^{142V}(*142V*). Sample immunoblots are shown above. Data are expressed as mean ± s.e.m. **P*<0.05, Student's *t*-test, *n*=3/group. (**E**) *AGTR1* promoter activity in hRPTCs transfected with *hGRK4*γ^{WT}(*WT*) or *hGRK4*γ^{142V}(*142V*) and empty vector (EV) and *hGRK2* (*GRK2*) as negative controls. Cells were co-transfected with pAT_1RLuc plasmid (which contains the human *AGTR1* promoter DNA from −1941 to +281 relative to the transcription start codon) and dual luciferase activity was measured 24 hr later. Data are expressed as mean ± s.e.m. **P*<0.05 *vs*. other groups, one-way ANOVA and Holm-Sidak test, *n*=4/group.

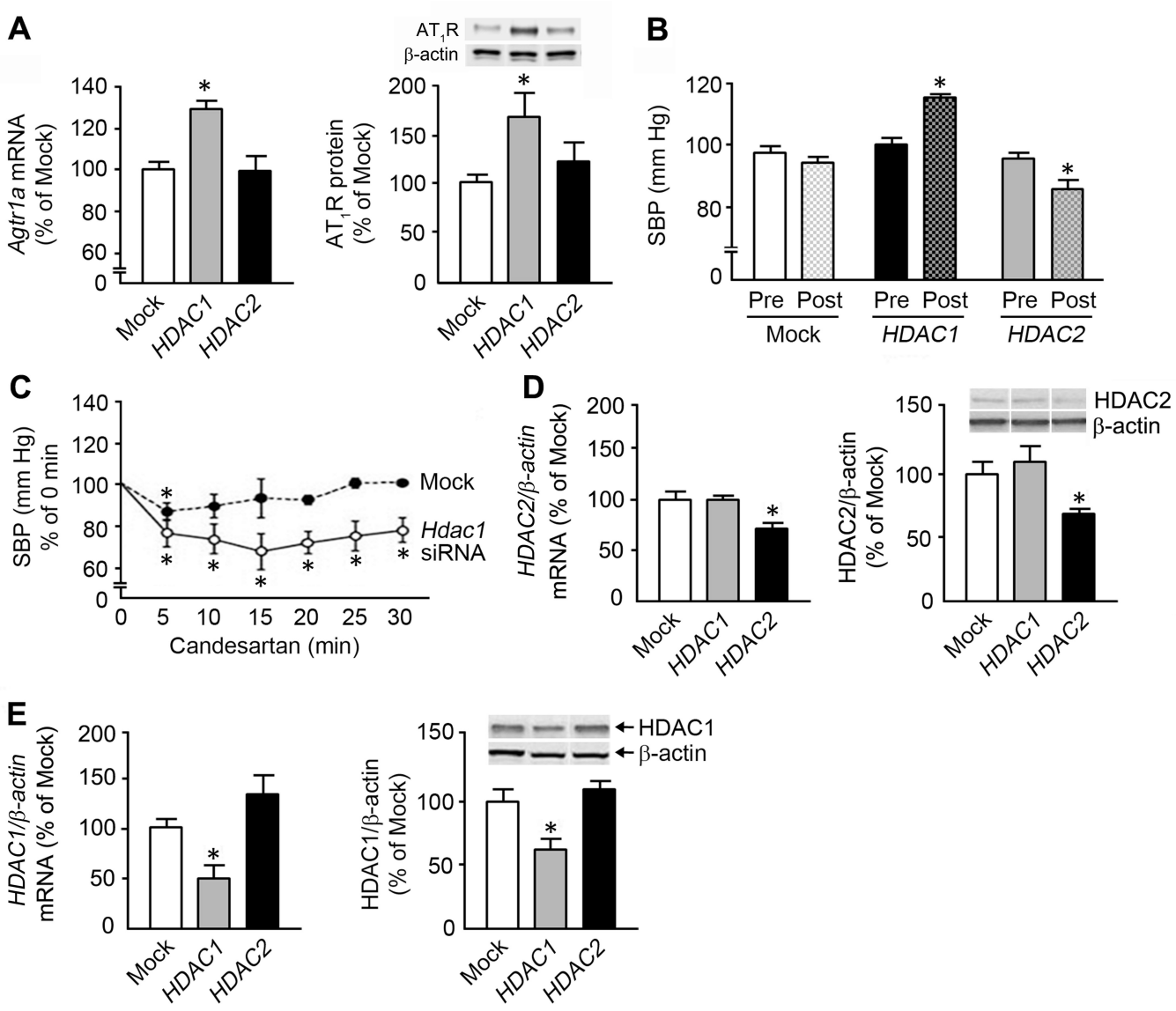

Figure 3. Effects of renal HDAC1 depletion on AT_1R expression and BP

Agtr1 mRNA and protein expression (**A**), and systolic BP (SBP) (**B**) in pentobarbital-anesthetized C57Bl/6 mice renally infused with siRNA against mouse *Hdac1* and *Hdac2*, or with non-silencing "Mock" siRNA. SBP (under pentobarbital anesthesia) was measured before (Pre) and after (Post) the 7-day siRNA infusion. Data are expressed as mean ± s.e.m. **P*<0.05 *vs*. other groups, one-way ANOVA and Holm-Sidak test, *n*=4/group. (**C**) SBP of pentobarbital-anesthetized C57Bl/6 mice infused with siRNA against *Hdac1*, or "Mock" siRNA as control, and treated with the AT_1R blocker candesartan. Data are expressed as mean ± s.e.m. and as percentage of basal value. Endogenous mouse HDAC2 (**D**) and HDAC1 (**E**) mRNA and protein expression in the kidney of C57Bl/6 mice chronically infused in the kidney with siRNA against mouse *Hdac2* or *Hdac1*, respectively, or with non-silencing "Mock" siRNA. β-actin was used as a housekeeping protein. Sample immunoblots are shown above. Data are expressed as mean ± s.e.m. **P*<0.05 *vs*. other groups, one-way ANOVA and Holm-Sidak test, *n*=5–6/group.

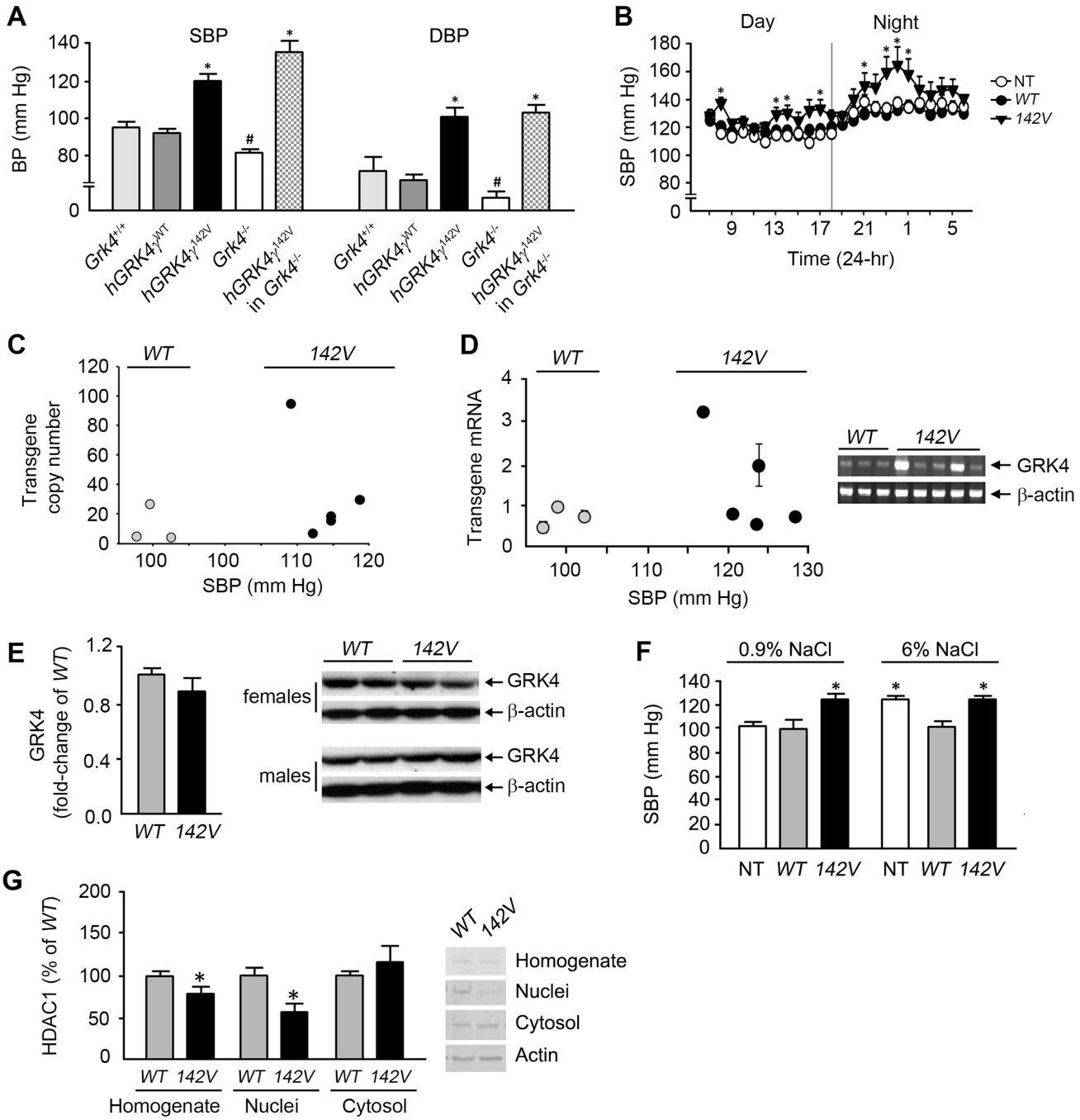

Figure 4. Effects of the *GRK4* on BP

(**A**) Systolic and diastolic BP (SBP and DBP, respectively) measured under pentobarbital anesthesia in $Grk4^{+/+}$ mice (n=26), mice expressing $hGRK4\gamma^{WT}$ (n=42) and $hGRK4\gamma^{142V}$ (n=54), $Grk4^{-/-}$ mice (n=5), and $Grk4^{-/-}$ mice expressing the human $GRK4\gamma^{142V}$ transgene (n=4). Data are expressed as mean ± s.e.m. $^{*}P<0.05$ and $^{\#}P<0.001$, *vs*. others, one-way ANOVA and Scheffe's test. (**B**) SBP measured via telemetry in conscious mice expressing $hGRK4\gamma^{142V}$ (n=10) or $hGRK4\gamma^{WT}$ (n=8), and in $Grk4^{+/+}$mice (n=4). Data are expressed as mean ± s.e.m. $^{*}P<0.05$, *vs*. others, one-way ANOVA and Scheffe's test. (**C**) Copy number

of the transgene and (**D**) number of transgene mRNA plotted against the SBP, measured under pentobarbital anesthesia, of mice expressing *hGRK4γ*^{WT} (*WT*) or *hGRK4γ*^{142V}(*142V*). (**E**) Expression of the total renal GRK4 protein in male and female mice expressing *hGRK4γ*^{WT}(*WT*) or *hGRK4γ*^{142V}(*142V*). β-actin was used as housekeeping protein. (**F**) SBP in mice expressing *hGRK4γ*^{WT} or *hGRK4γ*^{142V}, or non-transgenic (NT) littermates as another set of controls, on normal (0.9% NaCl) and high (6% NaCl) salt diet, n=5–9/group. (**G**) HDAC1 expression in whole kidney homogenates and nuclear and cytoplasmic fractions from transgenic mice expressing *hGRK4γ*^{WT}(*WT*) or *hGRK4γ*^{142V}(*142V*). Data are expressed as mean ± s.e.m. **P*<0.05, *vs*. others, one-way ANOVA and Holm-Sidak test, *n*=5/group.

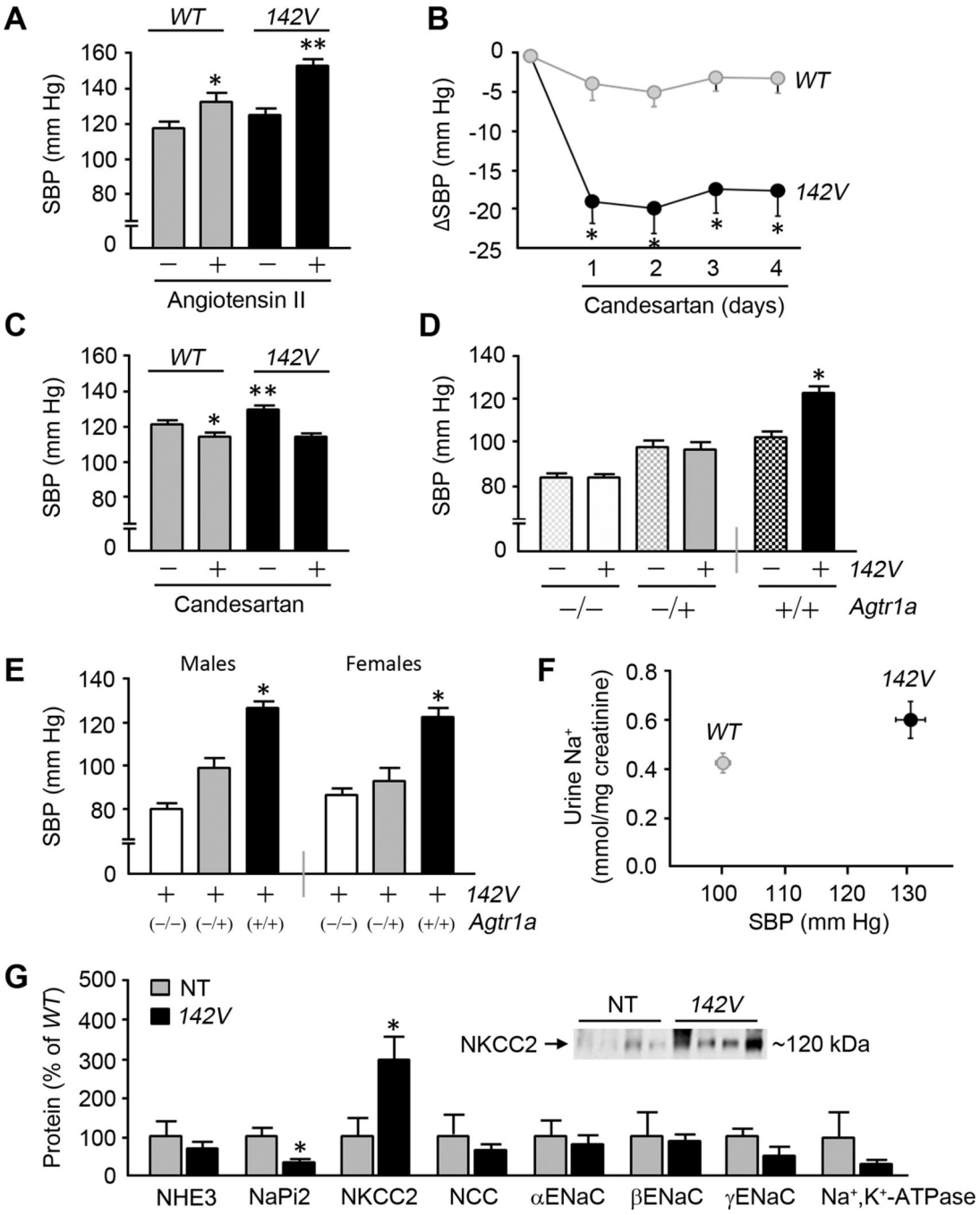

Figure 5. Effects of AT_1R agonism, blockade, or deficiency in $hGRK4\gamma^{WT}$ $hGRK4\gamma^{142V}$ mice

(**A**) Daytime systolic BP (SBP, measured via telemetry) in mice expressing $hGRK4\gamma^{WT}$(*WT*; n=9) or $hGRK4\gamma^{142V}$(*142V*; n=6) treated with AngII (+) or vehicle (−). Data are expressed as mean ± s.e.m. **P*<0.05, vs. WT treated with AngII (+), ***P*<0.05 vs. others, one-way ANOVA, Holm-Sidak test, *n*=6–9/group. (**B**) Decrease in SBP, measured by telemetry, after AT_1R blockade with candesartan in mice expressing $hGRK4\gamma^{WT}$(*WT*) or $hGRK4\gamma^{142V}$(*142V*). Data are expressed as mean ± s.e.m. **P*<0.05 vs 0 time and WT, one-way ANOVA and Holm-Sidak test, *n*=6–9/group. (**C**) Twenty four hr SBP response to

AT_1R blockade with candesartan measured via telemetry in mice expressing *hGRK4*γ^{WT}(*WT*) or *hGRK4*γ^{142V}(*142V*). Data are expressed as mean ± s.e.m. **P*<0.05, *vs*. (−) *WT*; ***P*<0.01, *vs*. others, one-way ANOVA and Holm-Sidak test, *n*=7/group. (**D**) SBP of offspring of pentobarbital-anesthetized *hGRK4*γ^{142V} (*142V*) and *Agtr1a*$^{-/-}$ mice, including *Agtr1a*$^{+/+}$/*142V* mice (*n*=54), *Agtr1a*$^{+/+}$ without *142V* mice (*n*=26), *Agtr1a*$^{+/-}$/*142V* mice (*n*=15) and *Agtr1a*$^{+/-}$ without *142V* littermates (*n*=13). Data are expressed as mean ± s.e.m. **P*<0.001, *vs*. others, one-way ANOVA and Scheffe's test. (**E**) SBP of pentobarbital-anesthetized male and female offspring of *hGRK4*γ^{142V} (*142V*) and *Agtr1a*$^{-/-}$ mice, including *Agtr1a*$^{+/+}$/*142V* mice (male=25, female=21), *Agtr1a*$^{+/-}$/*142V* mice (male=9, female=6) and *Agtr1a*$^{-/-}$/*142V* mice (male=2, female=3). Data are expressed as mean ± s.e.m. **P*<0.001, *vs*. others, one-way ANOVA and Holm-Sidak test. (**F**) A pressure vs. natriuresis plot for *hGRK4*γ^{WT}(*WT*) and *hGRK4*γ^{142V}(*142V*) mice; SBP measured under pentobarbital anesthesia. Data are expressed as mean ± s.e.m. (**G**) Protein expression of renal sodium transporters, pump (Na^+,K^+-ATPase, α subunit) and channels in plasma-membrane-enriched fractions from kidneys of mice expressing *hGRK4*γ^{142V}(*142V*) and non-transgenic littermates (NT). Data are expressed as mean ± s.e.m. **P*<0.01, *t*-test, *n*=4/group. A sample immunoblot of NKCC2 is shown.

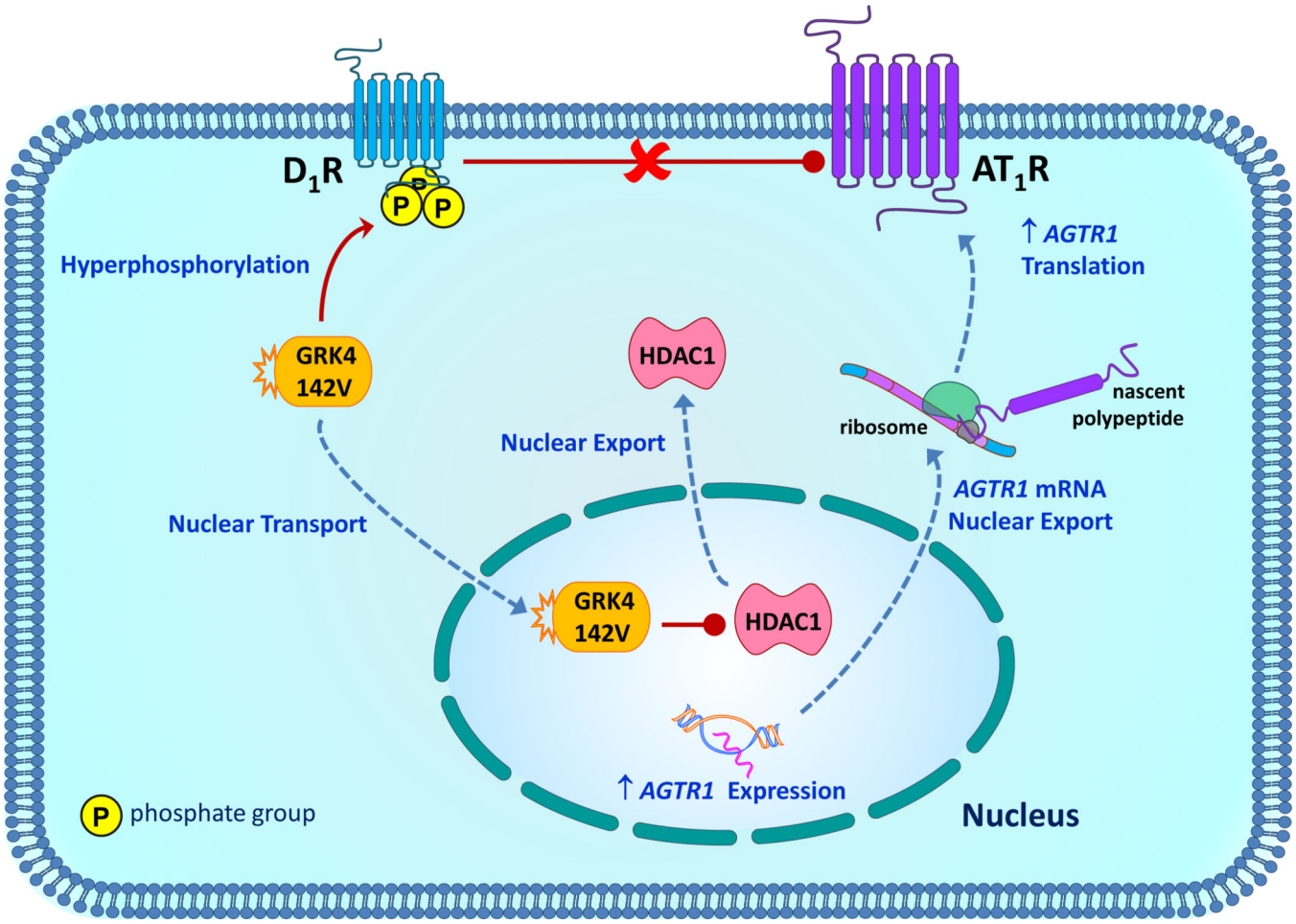

Figure 6. Renal mechanisms by which *GRK4*^{142V} promotes hypertension

Wild-type GRK4 is required for the normal activity of renal D_1R for three reasons: 1) GRK4 phosphorylates and desensitizes agonist-activated D_1R to temper the cellular response; 2) GRK4 aids in the recruitment of β-arrestins required for receptor kinase endocytosis; and 3) GRK4 participates in the proper orientation of D_1R at the plasma membrane. The GRK4γ^{142V} variant promotes hypertension via two mechanisms. First, GRK4γ^{142V} hyperphosphorylates the D_1R, rendering it unable to respond to subsequent stimulation, thus preventing the receptor kinase from inhibiting renal sodium transport. Since the D_1R and AT_1R negatively regulate one another, a dysfunction of the D_1R also results in enhanced AT_1R effect which, in concert with the inability of D_1R to inhibit renal sodium transport, leads to hypertension. D_1R promotes natriuresis (excretion of excess sodium), while AT_1R promotes anti-natriuresis. Second, owing to its nuclear localization signal, GRK4^{WT} may translocate to the nucleus and inhibit the *AGTR1* promoter activity. However, nuclear GRK4γ^{142V} allows the unfettered expression of *AGTR1* by inhibiting HDAC1 and promoting its nuclear export. This results in an open nucleosome conformation, increased AT_1R abundance and enhanced AT_1R effect, and eventually hypertension.