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Sorting nexin 1 loss results in increased oxidative stress and hypertension

Jian Yang[1], **Laureano D. Asico**[2,3], **Amber L. Beitelshees**[4], **Jun B. Feranil**[2], **Xiaoyan Wang**[2,3], **John E. Jones**[2], **Ines Armando**[2,3], **Santiago G. Cuevas**[2,3], **Gary L. Schwartz**[5], **John G. Gums**[6,7], **Arlene B. Chapman**[8], **Stephen T. Turner**[5], **Eric Boerwinkle**[9], **Rhonda M. Cooper-DeHoff**[6], **Julie A. Johnson**[6], **Robin A. Felder**[10], **Edward J. Weinman**[2,11], **Chunyu Zeng**[12,13], **Pedro A. Jose**[2,3,14], **Van Anthony M. Villar**[2,3]

[1]Department of Clinical Nutrition, The Third Affiliated Hospital of Chongqing Medical University, Chongqing, P.R. China

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

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

[4]Division of Endocrinology, Diabetes and Nutrition, Department of Medicine, Program for Personalized and Genomic Medicine, University of Maryland School of Medicine, Baltimore, MD, USA

[5]Division of Nephrology and Hypertension, Department of Internal Medicine, College of Medicine, Mayo Clinic, Rochester, MN, USA

[6]Department of Pharmacotherapy and Translational Research, Center for Pharmacogenomics, College of Pharmacy, University of Florida, Gainesville, FL, USA

[7]Department of Community Health and Family Medicine, College of Medicine, University of Florida, Gainesville, FL, USA

[8]Renal Division, Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA

[9]Human Genetics and Institute of Molecular Medicine, University of Texas Health Science Center, Houston, TX, USA

[10]Department of Pathology, The University of Virginia School of Medicine, Charlottesville, VA, USA

Correspondence Jian Yang, Department of Clinical Nutrition, The Third Affiliated Hospital of Chongqing Medical University, Chongqing 410020, P.R. China. jianyang@hospital.cqmu.edu.cn; Chunyu Zeng, Department of Cardiology, Fujian Heart Medical Center, Fujian Medical University Union Hospital, Fujian, 350001, P.R.China. chunyuzeng01@163.com.

AUTHOR CONTRIBUTIONS

J. Yang, C. Zeng, P. A. Jose, and V. A. M. Villar designed research; J. Yang, L. D. Asico, A. L. Beitelshees, J. B. Feranil, X. Wang, J. E. Jones, I. Armando, S. G. Cuevas, G. L. Schwartz, J. G. Gums, A. B. Chapman, S. T. Turner, E. Boerwinkle, R. M. Cooper-DeHoff, and J. A. Johnson performed the research; J. Yang, L. D. Asico, A. L. Beitelshees, R. A. Felder, E. J. Weinman, C. Zeng, P. A. Jose, and V. AM Villar analyzed data; J. Yang, C. Zeng, P. A. Jose, and V. A. M. Villar wrote the paper.

CONFLICT OF INTEREST

Drs. Jose and Felder own Hypogen, Inc, which owns the US Patent (6660474B1) for GRK4. All other authors have no conflict of interest.

[11]The Department of Veterans Affairs, Baltimore, MD, USA

[12]Department of Cardiology, Fujian Heart Medical Center, Fujian Medical University Union Hospital, Fujian, P.R.China

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

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

Abstract

Acute renal depletion of sorting nexin 1 (SNX1) in mice results in blunted natriuretic response and hypertension due to impaired dopamine D_5 receptor (D_5R) activity. We elucidated the molecular mechanisms for these phenotypes in *Snx1*$^{-/-}$ mice. These mice had increased renal expressions of angiotensin II type 1 receptor (AT_1R), NADPH oxidase (NOX) subunits, D_5R, and NaCl cotransporter. Basal reactive oxygen species (ROS), NOX activity, and blood pressure (BP) were also higher in *Snx1*$^{-/-}$ mice, which were normalized by apocynin, a drug that prevents NOX assembly. Renal proximal tubule (RPT) cells from hypertensive (HT) Euro-American males had deficient SNX1 activity, impaired D_5R endocytosis, and increased ROS compared with cells from normotensive (NT) Euro-American males. siRNA-mediated depletion of SNX1 in RPT cells from NT subjects led to a blunting of D_5R agonist-induced increase in cAMP production and decrease in Na^+ transport, effects that were normalized by over-expression of SNX1. Among HT African-Americans, three of the 12 single nucleotide polymorphisms interrogated for the *SNX1* gene were associated with a decrease in systolic BP in response to hydrochlorothiazide (HCTZ). The results illustrate a new paradigm for the development of hypertension and imply that the trafficking protein SNX1 may be a crucial determinant for hypertension and response to antihypertensive therapy.

Keywords

dopamine D_5 receptor; hypertension; oxidative stress; renal proximal tubule cells; sorting nexin 1

1 | INTRODUCTION

The kidney plays a vital role in the long-term regulation of blood pressure (BP). Na^+ balance, which is abnormally increased in hypertension, is tightly regulated by both natriuretic and anti-natriuretic hormones, for example, dopamine and angiotensin II (Ang II), respectively. These hormones exert their effects via G protein-coupled receptors (GPCRs).[1] Dopamine receptors are classified into the D_1-like (D_1R and D_5R) and D_2-like (D_2R, D_3R, and D_4R) subtypes based on their structure and pharmacology. The D_1-like receptors couple to the stimulatory G protein $G\alpha_S$ and stimulate adenylyl cyclase activity, whereas D_2-like receptors couple to the inhibitory G protein $G\alpha i$ and inhibit adenylyl cyclase activity.[1] A major portion of renal Na^+ handling is regulated by D_1-like dopamine receptors during conditions of moderate Na^+ excess.[2] Although the D_1R has received more attention than dopamine D_5 receptor (D_5R) in the pathogenesis of hypertension,[1,2] the D_5R has a higher affinity for dopamine than D_1R and exhibits constitutive activity.[3] D_5R also shows a

trafficking profile distinct from that of the other dopamine receptors.[4] The human D_5R gene *DRD5* locus at 4p15.1-16.1 is linked to essential hypertension.[5] The D_5R has regulatory effects, independent of D_1R, on cellular signal transduction, reactive oxygen species (ROS) production, renal Na^+ transport, and BP.[6-8] We have reported that disruption of the *Drd5* gene (*Drd5*$^{-/-}$) in mice results in hypertension, which is aggravated by high-salt diet.[9] The hypertension in *Drd5*$^{-/-}$ in mice is related, in part to increased renal expression of the Ang II type 1 receptor (AT_1R) and ROS production.[8,9] The renal D_5R is important in the maintenance of normal BP because the high BP of *Drd5*$^{-/-}$ mouse is decreased by the transplantation of wild-type kidney, whereas the normal BP of *Drd5*$^{+/+}$ mouse is increased by the transplantation of *Drd5*$^{-/-}$ kidney.[9]

The sorting nexin 1 (SNX) family is comprised of a diverse group of cytoplasmic- and membrane-associated proteins that are involved in various aspects of receptor endocytosis and trafficking through the endosomes.[10] SNX1 homodimerizes or heterodimerizes with SNX2 to form the membrane-targeting subunit of the mammalian retromer. The human SNX1 is comprised of an N-terminal SNX region, a central Phox homology (PX) domain, and a C-terminal Bin-Amphiphysin-Rvs (BAR) domain that binds to and/or induces membrane curvature via interactions with the plasma membrane lipid bilayer. SNX1 is involved in the sorting of cell surface receptors and GPCRs.[11,12]

SNX1 can clearly distinguish between the two D_1-like receptors, D_1R and D_5R; D_5R but not D_1R binds strongly with this protein.[13] We have reported that SNX1 is crucial for renal D_5R trafficking, signal transduction, and function in human renal proximal tubule cells (hRPTCs) and in C57Bl/6J and BALB/cJ mice, as demonstrated by impaired natriuretic response to D_1-like receptor agonist stimulation and development of hypertension after acute depletion of renal SNX1.[11] The aim of the current study is to elucidate the molecular mechanisms for these phenotypes in *Snx1*$^{-/-}$ mice and hRPTCs, including the production of ROS. We also investigated the association of *SNX1* single nucleotide polymorphisms (SNPs) with BP response to the β-adrenergic blocker atenolol and the diuretic hydrochlorothiazide (HCTZ) among hypertensive (HT) subjects from the Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) study.[14] Our results demonstrate that SNX1 is a crucial determinant in the pathogenesis of hypertension and illustrate a new paradigm for the development of this disorder.

2 | MATERIALS AND METHODS

2.1 | Generation of *Snx1*$^{-/-}$ Mice

Snx1$^{-/-}$ mice were generated by replacing the coding region of exon 1 and the following splice junction site with a modified gene cassette containing the selectable markers HIS3 and neomycin flanked by loxP site.[15] The mice were revived from cryopreservation and were used to establish a breeding colony at the University of Maryland School of Medicine Animal Facility. Adult (~28-wk old) male and female *Snx1*$^{-/-}$ mice and wild-type littermates, identified by DNA genotyping, were studied. The mice were maintained on normal salt (0.6% NaCl) diet, unless otherwise indicated.

2.2 | Study approval

All animals used in this study were bred and maintained at the animal facilities of the University of Maryland School of Medicine. The studies were conducted in accordance with NIH guidelines for the ethical treatment and handling of animals in research and approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee (IACUC).

2.3 | BP measurement by telemetry

BP was measured in conscious mice by telemetry. The carotid arteries of mice were fitted with TA11PA-C10 transmitters (Data Sciences International, St. Paul, MN) under isoflurane anesthesia. Three percent isoflurane was used for induction and 1% for maintenance of anesthesia. One week after the implantation of the transmitters, the BPs were measured every hour in conscious mice on individual receiver platforms[16] and recorded in a dedicated computer that ran and analyzed the data using Dataquest.

2.4 | BP measurement under anesthesia

The mice were housed in metabolic cages the day before BP measurement for the collection of 24-h urine samples. Under pentobarbital (50 mg/kg body weight, intraperitoneal injection) anesthesia, the femoral artery was cannulated using a PE-50 tube with the tip heat-stretched to 180 μm. The catheter was advanced to the lower abdominal aorta, and then, connected to a BP transducer (Cardiomax II; Columbus Instruments, Columbus, OH).[11,16] BP was recorded 1 hour after the induction of anesthesia and when the BP became stable.

2.5 | Renal clearance study

The mice were anesthetized with pentobarbital prior to cannulation of the femoral arteries (BP monitoring), femoral veins (infusion of fenoldopam), and jugular veins (loading of normal saline equivalent to 5% of the body weight and given over 30 minutes and fluid maintenance).[11] Another catheter was inserted through a cystostomy and secured onto the bladder wall for urine collection. Urine samples were collected every hour starting from baseline followed by a treatment period (fenoldopam, 2 μg/kg/min intravenously for 1 h) and finally, a recovery period. Urine Na^+ was analyzed using Synchron EL-ISE Electrolyte system (Beckman). UNaV was calculated as urine volume x Na^+ (mEq/L).

2.6 | Apocynin treatment

Mice were treated with apocynin (3 mg/kg/day for 10 days; Sigma-Aldrich, St. Louis, MO), a drug that prevents NOX assembly,[6] or vehicle as control, via an osmotic minipump infusion into the subcutaneous tissue. The mice were housed in metabolic cages for the collection of 24-h urine samples before installing the minipumps and again at the end of the treatment period. BPs were also measured under anesthesia at the start and end of each treatment period. At the end of the recovery period, the mice were sacrificed and the organs were harvested and stored at −80°C.

2.7 | Cell lines and cell culture

Immortalized hRPTCs obtained from normotensive (NT) and HT Euro-American males were grown in DMEM/F12 supplemented with 10% of fetal bovine serum, epidermal growth factor (EGF, 10 ng/mL), insulin, transferrin, selenium cocktail (5 μg/mL), and dexamethasone (4 ng/mL) at 37°C with humidified 5% CO_2 in 95% air. The hRPTCs used were limited to <20 passages to avoid the confounding effects of cellular senescence. The cells tested negative for *Mycoplasma* infection.

2.8 | *SNX1*-specific siRNA and plasmid transfection

hRPTCs from NT males were transfected with *SNX1*-specific siRNA using HiPerFect (Qiagen, Germantown, MD), while hRPTCs from HT males were transfected with pcDNA3.1-*SNX1* plasmid using Fugene (Promega, Madison, WI). HiPerFect and Fugene are ideal for transfecting siRNA and DNA plasmids, respectively. Non-silencing siRNA and pcDNA3.1 (empty vector) were used as negative controls. After 72 hours, the cells were harvested for cAMP assay and Na^+ transport studies.

2.9 | cAMP accumulation assay

hRPTCs, transfected with either *SNX1*-specific siRNA or pcDNA3.1-*SNX1* plasmid, were grown in 12-well plates. Three days post-transfection, SNX1-depleted and -rescued hRPTCs were treated with fenoldopam (1 μM, 30 min) after pretreatment with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 μM; Calbiochem, Billerica, MA). The intracellular cAMP levels were quantified using the DetectX Direct cAMP assay (Cayman Chemical, Ann Arbor, MI) and the protein concentrations were quantified using a BCA Protein Assay kit (Thermo Scientific, Rockford, IL). The cAMP data were normalized for protein concentration.

2.10 | Cellular Na^+ transport studies

SNX1-depleted and -rescued hRPTCs were grown to confluence under polarized conditions on Corning HTS Transwell supports in 12-well plates and incubated at 37°C in an incubator with humidified 5% CO_2 in 95% air. The hRPTCs were serum-starved for 2 hours and treated with fenoldopam (1 μM/15 min) or vehicle (PBS), as control, at the bottom chamber (basolateral side). After washing, the hRPTCs were incubated with the permeant Na^+ indicator Na^+ green tetraacetate (5 μM; Molecular Probes, Eugene, OR) in complete, phenol red-free medium. The fluorescence signal, indicating the intracellular Na^+ level, for each Transwell was measured using the VICTOR3 V Multilabel Counter (485-nm excitation, 535-nm emission; Perkin-Elmer, Shelton, CT). Ouabain (50 μM, 1 h) added to the bottom chamber was used to inhibit Na^+-K^+-ATPase (NKA) activity.

2.11 | NKA activity measurement

NKA activity in the crude membrane fraction was measured using ouabain to inhibit NKA activity, as previously described.[17] NKA activity was calculated as the difference between total ATPase activity and ouabain-insensitive ATPase activity, which was corrected for protein concentration.

2.12 | Immunoblotting

Whole kidney homogenates, total cell lysates, and plasma membrane and cytoplasmic fractions were subjected to immunoblotting, as previously reported.[11] In brief, similar amounts of protein were resolved via 10% of SDS-PAGE and electro-transferred onto nitrocellulose membranes. Thereafter, the transblots were first probed with the primary antibody and subsequently the corresponding secondary antibody. The immunoreactive bands were visualized by chemiluminescence and autoradiography or the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE). The primary antibodies were purchased from GeneTex (D_5R), Origene (D_1R), Abcam (3-nitrotyrosine), Santa Cruz Biotechnology (AT_1R, D_2R, SNX2, SNX6, clathrin, HO-1, NOX1, NOX2, $p67^{phox}$), ProteinTech (SNX1, SNX5), Abbiotec (β-arrestin), BD Biosciences (IR-β), Chemicon (LEPR), Millipore (NKA, $p47^{phox}$, GAPDH), Bioworld ($p22^{phox}$, α_{1A}-AR), and Stressgen Biotechnologies (HO-2, SOD1, SOD2, SOD3). Some antibodies used are proprietary and previously characterized (D_1R, D_3R, D_4R, ETBR, NOX4).[18-21] The antibodies against NHE3, thiazide-sensitive NaCl cotransporter (NCC), and NKCC2 were gifts from Dr Mark A. Knepper (Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health). These antibodies have been validated.[16,18-20]

2.13 | Confocal microscopy

hRPTCs were grown to 50% confluence on poly-D-lysine-coated coverslips (BD Biosciences, San Jose, CA) in 24-well plates. The cells were serum-starved for 1 hour before treatment with fenoldopam (1 μM) at the indicated time points. The cells were immunostained with rabbit anti-D_5R (GeneTex) and donkey anti-rabbit secondary antibody tagged with Alexa Fluor 488 (Molecular Probes). The plasma membrane and nucleus were labeled using cholera toxin subunit B (CTxB) conjugated with Alexa Fluor 647 (Molecular Probes) and DAPI, respectively. Images were obtained with an LSM 510 DUO microscope with 63/NA1.4 oil-immersion objective (Zeiss, Thornwood, NY). The images were processed using Zen 2011 software (Zeiss).

2.14 | Measurement of ROS

ROS in the renal homogenates were assayed through the oxidation of 2′,7′-dichlorofluoresceindiacetate (DCFDA; Molecular Probes). Briefly, renal homogenates were incubated with fresh DCFDA (10 μM) for 30 minutes at 37°C. DCFDA fluorescence was measured using a microplate reader in 96-well plates at 485-nm excitation and 530-nm emission wavelengths. ROS production was expressed in arbitrary units corrected for protein concentration. All assays were performed in duplicate.

2.15 | NADPH oxidase activity

NADPH oxidase (NOX) activity was measured using lucigenin chemiluminescence in the presence of NADPH (100 μM; ICN Biomedicals, Irvine, CA). Equal amounts of whole kidney homogenates were incubated with lucigenin (5 μM; Invitrogen) for 10 minutes at 37°C in a final volume of 200 μl of assay buffer. NOX activity was expressed as arbitrary units corrected for the protein concentration. All assays were performed in duplicate.

2.16 | Measurement of reactive nitrogen species and lipid peroxidation

3-nitrotyrosine (marker of reactive nitrogen species) in kidney samples was quantified by immunoblotting (Abcam, Cambridge, MA). The levels of malondialdehyde-adducts (MDA-adducts) (marker of lipid peroxidation) in kidney samples were measured using a commercially available kit (OxiSelect MDA Adduct ELISA Kit; Cell Biolabs, San Diego, CA).

2.17 | PEAR study

Details of the Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) study have been published.[14] Briefly, PEAR-enrolled patients with essential hypertension were randomized into atenolol or HCTZ treatment groups. After a washout period of approximately 4 weeks, the patients were treated as per protocol for an average of 9 weeks. The BP response to monotherapy was assessed using a composite of home, ambulatory, and clinic BP measurements before the "other" drug was added on for another 9 weeks and response to the dual therapy was assessed similarly. For the purpose of this study, we focused on the BP response to atenolol and HCTZ monotherapy in 227 Euro-Americans and 147 African-American patients. Genotypes were determined using Illumina Human Omni1-Quad BeadChip (Illumina, San Diego CA). Genotype imputation was performed using MaCH software program (version 1.0.16) with SNPs that passed quality-control filtering and HapMap III-phased haplotypes as reference panel. *SNX1* SNPs were defined as those ± 5 kb from *SNX1* (n = 12 SNPs).

2.18 | Statistical analysis

Numerical data are expressed as mean ± standard error of the mean (SEM). Significant difference between two groups was determined by Student's *t* test, while that among three or more groups was determined by one-way ANOVA and Holm-Sidak post hoc test. $P < .05$ was considered significant. Statistical analysis was performed using SigmaStat 3.5 (Richmond, CA). BP response to atenolol or HCTZ in PEAR was compared by *SNX1* genotype with an additive model using linear regression adjusted for age and sex in Euro-Americans or African-Americans. $P < .01$ was considered significant given the linkage disequilibrium between the SNPs. Hardy Weinberg Equilibrium (HWE) was assessed within each race group using chi-square test and one degree of freedom. Analyses were performed using PLINK (http://pngu.mgh.harvard.edu/purcell/plink/).

3 | RESULTS

3.1 | *Snx1*$^{-/-}$ mice have impaired natriuretic and BP responses to D_1-like receptor stimulation

We have reported that the acute siRNA-mediated, renal-restricted depletion of SNX1 in salt-sensitive C57Bl/6J or salt-resistant BALB/cJ mice resulted in impaired D_5R-dependent Na^+ excretion and high BP.[11] To determine further the underlying mechanisms involved in the development of hypertension in the absence of SNX1, we studied *Snx1*$^{-/-}$ mice (Figure 1A).[15] These mice, on a 129S1/SvlmJ genetic background, have germline deletion of *Snx1*. Since the renal-restricted depletion of *Snx1* in adult mice perturbs the activity of agonist-

activated D_5R,[11] we first studied the response of Na^+-loaded adult mice to the D_1R/D_5R agonist fenoldopam; there is no commercially available agonist that can distinguish D_1R from D_5R. The intravenous administration of a non-hypotensive dose of fenoldopam (2 μg/kg/min/1 h)[16] increased Na^+ excretion in wild-type mice (UNaV = 201.5 ± 40.1% vs 100 ± 17%, basal) but not in *Snx1*$^{-/-}$ littermates (UNaV = 83±11.5% vs 100 ± 20.7%, basal) (Figure 1B). *Snx1*$^{-/-}$ mice had elevated systolic (131.3 ± 6.4 mm Hg vs 105.5 ± 6.4, controls) and diastolic (107.3 ± 5.3 mm Hg vs 84.2 ± 9, controls) BPs measured under pentobarbital anesthesia (Figure 1C,D), reproducing the increased BP of mice with renal-restricted depletion of SNX1.[11] We next subjected another group of mice fed low (<0.02% NaCl), normal (0.6% NaCl), or high-salt (6.0% NaCl) diet to determine if the BP can be influenced by the amount of dietary Na^+. The *Snx1*$^{-/-}$ mice had elevated nocturnal and daytime BP (measured by telemetry), regardless of dietary salt intake, compared with wild-type littermates (Figure 1E). The BPs of *Snx1*$^{-/-}$ mice were not affected by salt intake but high-salt diet increased the nocturnal BP of wild-type littermates. Since mice are nocturnally active animals, the daytime BP was lower than the nighttime BP in both mouse strains but both nocturnal and diurnal BPs were higher in *Snx1*$^{-/-}$ mice than their wild-type littermates.

3.2 | *Snx1*$^{-/-}$ mice have increased renal protein expression of D_2R, D_3R, D_5R, AT_1R, NKA, NHE3, and NCC

We confirmed the absence of SNX1 in the *Snx1*$^{-/-}$ mice by immunoblotting of whole kidney homogenates (Figure 2A). Since SNX1 and SNX2 are reported to be functionally redundant,[15] we also studied the renal SNX2 expression and found that it was upregulated (123.3 ± 7% vs 100 ± 4.4%) in *Snx1*$^{-/-}$ mice, conceivably as a compensatory response. Because SNX1 has been shown to co-immunoprecipitate with SNX5 and SNX6,[22] we also evaluated the renal abundance of these proteins and found that there was no difference in the expression of SNX5 and SNX6 between the two mouse groups. *Snx1*$^{-/-}$ mice had increased levels of β-arrestin (115.2 ± 3.6% vs 100 ± 2.3%), but not clathrin, which are proteins involved in the endocytosis of agonist-activated receptors.[4,18] We next evaluated the expression profiles of some GPCRs that are implicated in BP regulation. *Snx1*$^{-/-}$ mice had elevated levels of renal D_5R (142.9 ± 4.7% vs 100 ± 6.8%), as well as D_2R (110 ± 2% vs 100 ± 0.8%) and D_3R (134.3 ± 5.3% vs 100 ± 1.2%) (Figure 2B), conceivably as compensatory mechanisms. D_1R and D_4R protein expressions were not different between *Snx1*$^{-/-}$ mice and their wild-type littermates.

Snx1$^{-/-}$ mice, relative to their wild-type littermates, had increased AT_1R (123.8 ± 2% vs 100 ± 2%) protein and decreased leptin receptor (LEPR) (75.4 ± 8.1% vs 100 ± 1.7%) protein, while ETBR, α_{1A}-adrenoceptor (α_{1A}-AR), and insulin receptor (IR-β) proteins were not different between the two mouse strains (Figure 2C). The increase in AT_1R protein mirrored the increase in renal AT_1R protein in mice with acute renal-restricted depletion of SNX1.[11]

Snx1$^{-/-}$ mice, relative to wild-type littermates, had increased renal protein expressions of NKA (121.2 ± 3.8% vs 100 ± 2.3%), Na^+-hydrogen exchanger 3 (NHE3 aka SLC9A3) (127 ± 11.1% vs 100 ± 3%), and thiazide-sensitive NaCl cotransporter (NCC aka SLC12A3) (119.2 ± 1% vs 100 ± 1%) proteins, but not the Na^+-potassium 2 chloride cotransporter

(NKCC2 aka SLC12A1) (Figure 2D). The increase in NCC protein mirrors the change observed in *Drd5*$^{-/-}$ mice.[7]

3.3 | *Snx1*$^{-/-}$ mice have increased renal protein expression of NOX components

Both D_5R and D_2R have antioxidant properties;[6,23] we have reported that the D_5R, expressed in the kidney, negatively regulates the expression of NOX components NOX2 (aka gp91^{phox}), NOX4, and p47^{phox}.[24,25] Therefore, we determined the protein expression profiles of the components of the NOX complex and found that NOX1 (153.4 ± 12.2% vs 100 ± 4.1%), NOX2 (129.8 ± 5.5% vs 100 ± 7.7%), and p47^{phox} (118.2 ± 2.7% vs 100 ± 5%) proteins in the kidney were upregulated in *Snx1*$^{-/-}$ mice, compared with their wild-type littermates, reflecting the phenotypes observed in *Drd5*$^{-/-}$ mice[24] (Figure 3A). Among the proteins involved in the protection against ROS, only paraoxonase 2 (PON2) (131.5 ± 11% vs 100 ± 1.9%) was upregulated in the kidney of *Snx1*$^{-/-}$ mice (Figure 3B). The protein expression of the three isoforms of superoxide dismutase (SOD) was not different between the two mouse strains. Although the D_5R exerts its antioxidant activity partly via heme oxygenase 1 (HO-1)[25] and increased levels of HO-1 ameliorates renovascular hypertension,[26] we did not observe any change in its expression; HO-2 was also unchanged. We also evaluated the presence of enhanced tyrosine nitration, which is a marker of nitrosative stress and inflammation and found that the *Snx1*$^{-/-}$ mice had higher basal renal levels of 3-nitrotyrosine (143.7 ± 11% vs 100 ± 4.8%) (Figure 3C). MDA, a marker of lipid peroxidation (see below) was also higher in *Snx1*$^{-/-}$ mice than their wild-type littermates.

3.4 | Apocynin reverses the oxidative stress and hypertension in *Snx1*$^{-/-}$ mice

NOX is the major source of ROS and several components (NOX1, NOX2, and p47^{phox}) (Figure 3A) of this enzyme complex were upregulated in *Snx1*$^{-/-}$ mice. Therefore, we measured the amount of ROS in renal tissues in mice treated with the chronic (10 days) subcutaneous infusion of apocynin, a drug that prevents the assembly of the NOX complex by preventing the translocation of p47^{phox} to NOX2.[6,27] *Snx1*$^{-/-}$ mice had markedly increased level of ROS (218.6 ± 7.7% vs 100 ± 17.9%), which was ameliorated by apocynin treatment (125.8 ± 20.4%) (Figure 4A). Apocynin did not change the renal ROS level in wild-type littermates. We also found that the renal NOX activity was higher in the *Snx1*$^{-/-}$ mice than their with wild-type littermates (43.9 ± 3.3 AU/mg protein/min vs 25.98 ± 3.5) (Figure 4B). Apocynin reduced the elevated renal NOX activity in *Snx1*$^{-/-}$ mice to the basal level of wild-type littermates (25.83 ± 4.3 AU/mg protein/min) but had no effect in wild-type littermates. Compared with wild-type littermates, *Snx1*$^{-/-}$ mice had increased renal level of MDA (32.5 ± 3.4 pmol/mg protein vs 17.2 ± 2.1), a marker of lipid peroxidation, which was normalized by apocynin treatment (16.6 ± 1.8). Apocynin did not affect the renal MDA level in wild-type littermates (14.4 ± 2.3) (Figure 4C). *Snx1*$^{-/-}$ mice had higher 3-nitrotyrosine level, a marker of nitrosative stress, than their wild-type littermates (128.3 ± 4% vs 100 ± 2%), which was normalized by apocynin (108 ± 3) (Figure 4D). Apocynin did not change the elevated renal expression of NOX2 or AT_1R in *Snx1*$^{-/-}$ mice (Figure 4E,F). Na^+ excretion corrected by urinary creatinine tended to be lower in *Snx1*$^{-/-}$ mice than their wild-type littermates but did not reach statistical significance (Figure 4G). Apocynin did not change the Na^+ excretion/creatinine in wild-type (2.08 ± .014 vs 2.22 ± 0.38) and *Snx1*$^{-/-}$ (1.61 ± 0.15 vs 1.45 ± 0.05) mice (Figure 4G). The *Snx1*$^{-/-}$ mice had higher systolic and

diastolic BPs than their wild-type littermates which had normal BP (Figure 4H). Apocynin normalized both the systolic (pretreatment: 131.3 ± 4.8 mm Hg vs posttreatment: 105.7 ± 0.3) and diastolic (pretreatment: 95.33 ± 5.8 mm Hg vs posttreatment: 74.4 ± 1.4) BPs of *Snx1*$^{-/-}$ mice, but did not change the BP of wild-type littermates, indicating a direct causal relationship between increased oxidative stress and increased BP in *Snx1*$^{-/-}$ mice. The failure of *Snx1*$^{-/-}$ mice to increase Na^+ excretion in the face of increased BP, that is, defective pressure-natriuresis, could have contributed to their increased BPs.

3.5 | Renal proximal tubule cells (RPTCs) from HT humans are SNX1-deficient

In order to determine if our observations in mice could be replicated in humans, we used several lines of immortalized hRPTCs obtained from NT and HT Euro-American males.[28] hRPTCs from HT subjects had reduced mRNA and protein expressions of SNX1, compared with hRPTCs from NT subjects (60 ± 2.1% vs 100 ± 10.2%, SNX1 protein) (Figure 5A), but had similar D_5R mRNA and protein expression (Figure 5B). hRPTCs from HT subjects had increased level of ROS (182 ± 10.5% vs 100 ± 11.4%) (Figure 5C), indicating that these cells were in a state of oxidative stress. To confirm the causal relationship between SNX1 depletion in the development of oxidative stress, we silenced *SNX1* gene in hRPTCs from NT subjects and found that the *SNX1*-depleted cells had increased ROS production (160 ± 18.3% vs 100 ± 3.4%) (Figure 5D). Because the total cellular expression of D_5R in hRPTCs was not different between NT and HT subjects (Figure 5B), we determined if the subcellular localization of D_5R is different between these subjects. Confocal microscopy showed that the D_5R was distributed to a large extent at the plasma membrane in hRPTCs from NT and HT subjects. The 15 minutes incubation of the hRPTCs from NT subjects with fenoldopam (1 μM), a D_1R, and D_5R agonist, caused the internalization and accumulation of D_5R at the juxta-nuclear area, especially at 15 minutes; a minimal effect was noted in hRPTCs from HT subjects which are SNX1-deficient (Figure 5E), indicating impaired D_5R trafficking in hRPTCs from HT subjects.

To determine a functional corollary to the tendency of *Snx1*$^{-/-}$ mice to have decreased Na^+ excretion, a failure of Na^+ excretion to increase with BP elevation (ie, pressure-natriuresis) (Figure 4G) or to the intravenous infusion of fenoldopam (Figure 1B), we studied the ability of the D_1R/D_5R agonist, fenoldopam (1 μM/15 min), to stimulate cAMP production and inhibit basolateral Na^+ transport in hRPTCs from NT and HT subjects. The ability of fenoldopam to increase cAMP can be related to its ability to decrease RPTC Na^+ transport via both cAMP and phospholipase C pathways.[1,29] We found that, as expected, fenoldopam increased cAMP production in hRPTCs from NT and minimally in hRPTCs from HT subjects (Figure 5F). The siRNA-mediated *SNX1* silencing in hRPTCs from NT subjects prevented the ability of fenoldopam to increase cAMP production. By contrast, *SNX1* gene rescue in hRPTCs from HT subjects almost completely restored the ability of fenoldopam to increase cAMP production to that observed in NT subjects (Figure 5F). The lack of cAMP response to the D_1R/D_5R agonist fenoldopam in cells with SNX1 deficiency, and hence D_5R dysfunction, in spite of the presence of functional D_1R, may be due to the requirement for the D_5R and D_1R to heterodimerize for a normal cAMP response.[29]

The studies in Figure 5F showing the importance of SNX1 in the positive cAMP response to D_1R/D_5R agonist fenoldopam stimulation provided the rationale to study the importance of SNX1 to renal Na^+ transport. We measured NKA activity in the hRPTCs grown in polarized conditions in Transwells. Under normal conditions, fenoldopam (1 μM/15 min) treatment at the basolateral side should activate the D_1R/D_5R to inhibit NKA activity, leading to an increase in intracellular Na^+. In hRPTCs from NT subjects, fenoldopam treatment increased cytosolic Na^+ (119.3 ± 4% vs 100 ± 2%), but not in hRPTCs from NT subjects depleted of their endogenous SNX1 (101.2 ± 1.9% vs 101.4 ± 7.1%) (Figure 5G). By contrast, fenoldopam treatment failed to increase the intracellular Na^+ in SNX1-deficient hRPTCs from HT subjects (92.6 ± 2.2% vs 100 ± 4%), but increased intracellular Na^+ (115 ± 4% vs 98.4 ± 1.7%) with reconstitution of SNX1 in the hRPTCs from HT subjects. Because SNX1 regulates D_5R but not D_1R function,[11,13] these data confirm the need for SNX1 for normal D_5R activity in hRPTCs and underscore the relevance of SNX1 in human renal physiology.

To determine if ROS, via NOX, are involved in the impaired ability of D_5R to inhibit NKA activity, we studied the effect of VAS2870, a specific NOX inhibitor.[30] Our results showed that the specific NOX inhibitor VAS2870, by itself, had no effect on ROS production, but prevented the increase in ROS production induced by *SNX1* depletion (Figure 5H). We also found that VAS2870, by itself, had no effect on fenoldopam-induced inhibition of NKA activity in control hRPTCs from NT Euro-Americans. However, VAS2870 treatment normalized the impaired fenoldopam-mediated inhibition of NKA activity in *SNX1*-specific siRNA-treated hRPTCs (Figure 5I). These results indicate that NOXs are involved in the increased ROS production and impaired fenoldopam-mediated inhibition of NKA activity in *SNX1* depleted hRPTCs.

3.6 | African-American carriers of some *SNX1* SNPs respond to the antihypertensive effect of HCTZ

To demonstrate further the clinical importance of SNX1 in hypertension, we evaluated 12 SNPs located within 5 kb of the human *SNX1* gene as possible genetic predictors of BP response to monotherapy using the diuretic drug HCTZ and the β-adrenergic receptor blocker atenolol among HT patients enrolled in the PEAR study.[14] All 12 SNPs were present in African-Americans and 10 were present in Euro-Americans. All genotype frequencies were in Hardy-Weinberg equilibrium ($P > .07$). The minor allele frequencies and betas for BP response to HCTZ are shown in Table 1. None of the SNPs was associated with BP response to HCTZ in Euro-Americans. In African-Americans, rs11635627 (CG and GG) and rs11854249 (CC) were associated with a significant decrease in systolic BP with HCTZ ($P = .013$-0.009). rs12591947, which is in strong linkage disequilibrium ($r^2 = 0.99$) with rs116535627, (CC and AC) trended toward significance ($P = .02$). Results for diastolic BP were in the same direction as the systolic BP but did not reach significance (Figure 6). None of the SNPs was associated with response to atenolol in either racial group.

4 | DISCUSSION

4.1 | Loss of SNX1 results in increased oxidative stress, impaired natriuresis, and hypertension

The D_5R plays a crucial role in the maintenance of normal BP.[1,6-9,24,25] This regulatory function is carried out in a number of ways in renal and extra-renal tissues. Foremost of these is the ability of renal D_5R to inhibit a number of Na^+ transporters and the Na^+ pump along the nephron, resulting in increased urinary Na^+ excretion when Na^+ intake is increased. The D_5R is expressed in almost all segments of the nephron[7] and heterodimerizes with the D_1R[29] to promote natriuresis. Second, the renal D_5R regulates BP by promoting the proteasomal degradation of the hypertensinogenic AT_1R,[8] in addition to its degradation via the lysosomes, as part of normal receptor turnover. Third, the D_5R, in conjunction with the D_1R, suppresses the expression and activity of the α_{1A}-adrenoceptor (α_{1A}-AR),[31] a receptor with anti-natriuretic and hypertensinogenic properties. Fourth, renal D_5R prevents oxidative stress by suppressing pro-oxidant activity (eg, NOX expression and activity) and enhancing antioxidant activity (eg, HO-1).[6,25] Moreover, the D_5R may curtail the development of oxidative stress by inhibiting the activity of phospholipase D (PLD) and increasing the activity of protein kinase A (PKA) in renal and vascular tissues.[6,32] The D_5R may also promote vasorelaxation of coronary smooth muscle cells by activating the large-conductance, calcium- and voltage-activated potassium (BKCa) channels.[33] In the absence of SNX1, these protective actions of D_5R are lost and an unfettered increase in BP ensues.

Many of the phenotypes observed in the *Snx1*$^{-/-}$ mice appear to be related to dysfunction of the D_5R. Although the SNX1-homolog SNX2 is upregulated, there is no reconstitution of renal D_5R activity since SNX2 and D_5R do not interact.[11] The β-arrestins are also upregulated, perhaps because agonist-activated D_5R requires them for endocytosis.[4] The D_5R is upregulated in the *Snx1*$^{-/-}$ mice kidneys, but not in *Snx1*-acutely depleted mice kidneys,[11] presumably an attempt to offset the impaired renal D_5R trafficking and activity in the long-term. The expression of D_2R and D_3R are both increased in the *Snx1*$^{-/-}$ mice. The D_2R[23] and D_5R[6,24,25] are bestowed with antioxidant properties and actively combat oxidative stress,[24] in part, through activation of PON2, HO-2, and DJ-1 for D_2R[23] and HO-1[25] and inhibition of NOX[6,24] for D_5R. The increased D_3R expression in *Snx1*$^{-/-}$ mice may be an attempt to compensate for the inability of D_5R[34] to decrease the increased renal tubular NKA expression and presumably activity in *Snx1*$^{-/-}$ mice. AT_1R, a receptor that has both hypertensinogenic and pro-oxidant activities, is upregulated in the kidney of *Snx1*$^{-/-}$ mice that may be due to the inability of D_5R to promote AT_1R degradation via the proteasome,[8] despite the increased D_3R expression.[35] The renal LEPR is decreased in *Snx1*$^{-/-}$ mice, which would go along with the reduction of renal LEPR in HT rats.[36]

Under normal conditions, ROS play an important role in the regulation of renal function such as Na^+ transport, renovascular tone, and tubuloglomerular feedback. For example, ROS increase Na^+ reabsorption in the thick ascending limb of the loop of Henle and renal medullary blood flow.[37] However, increased ROS production can cause abnormal kidney function, as in the case of hypertension and diabetes. NOX expression is elevated in the kidney of spontaneously HT rats[37] and *Drd5*$^{-/-}$ mice,[24] while NOX activity is increased in

diabetic nephropathy.[38] The *Snx1*$^{-/-}$ mice are under a general state of oxidative stress; NOX1, NOX2, and p47^{phox} are all upregulated in these mice. The antioxidant effect of apocynin is, in part, due to suppression of NOX activity.[6] Indeed, the present study shows that the chronic subcutaneous infusion of apocynin ameliorates the high BP in *Snx1*$^{-/-}$ mice, high-lighting the causal role of oxidative stress in the etiology of hypertension in these mice. We did not observe a change in AT_1R expression after apocynin treatment in *Snx1*$^{-/-}$ mice, although Liu et al reported that apocynin reverses the increase in AT_1R expression induced by Ang II in a rabbit neuronal cell line.[39] This may be due to the fact that the increase in AT_1R abundance in the *Snx1*$^{-/-}$ mice is not due to increased synthesis but rather to diminished proteasomal degradation of the receptor,[8] a process where apocynin plays no role. The antioxidant PON2 is upregulated, conceivably caused by the upregulation of D_2R, [23] in the kidney of *Snx1*$^{-/-}$ mice to counter-balance the oxidative stress in these mice.

The high BP of *Drd5*$^{-/-}$ and *Snx1*$^{-/-}$ mice is normalized by apocynin treatment. However, while the high BP in *Drd5*$^{-/-}$ mice is aggravated by high-salt diet, the BP in *Snx1*$^{-/-}$ mice is impervious to changes in dietary salt intake, even to low-salt diet. The reason for the discrepancy between the effect of Na^+ in the hypertension in *Drd5*$^{-/-}$ mice and *Snx1*$^{-/-}$ mice is currently unknown. *Drd5*$^{-/-}$ and *Snx1*$^{-/-}$ mice are on the C57Bl/6J[16] and 129S1/SvlmJ (unpublished data) genetic background, respectively, both of which can confer salt sensitivity,[16] precluding genetic background as the reason for the difference in the salt sensitivity between *Drd5*$^{-/-}$ and *Snx1*$^{-/-}$ mice. C57Bl/6J mice have increased renal AT_1R expression on high-salt diet similar to that of salt-resistant SJL mice.[16] C57BL/6J mice have only one renin gene and have lower BP than 129 mice that have two renin genes but AT_1R blockade decreases BP to a similar extent in these two mouse strains[40] and hence could not be a reason for the disparity in the salt sensitivity of BP. However, there is a difference in renal vascular lesions (eg, juxtaglomerular apparatus hypertrophy) with *Agtr1a* deletion in C57BL/6J and 129 mice that may be due to a modifier locus in chromosome 3.[41] Whether or not these gene modifiers could explain the difference in salt sensitivity between *Drd5*$^{-/-}$ and *Snx1*$^{-/-}$ mice remains to be determined.

Could the ability of SNX1 to interact with other receptors help explain the salt-sensitive phenotype in *Drd5*$^{-/-}$ but not *Snx1*$^{-/-}$ mice? SNX1 interacts with the epidermal growth factor receptor (EGFR), insulin receptor, and long form of LEPR. SNX1 enhances the degradation of EGFR; the absence of SNX1 may result in increased EGFR abundance, which in turn, would enhance renal proximal tubular fluid reabsorption.[42] The renal collecting duct ablation of the insulin receptor in mice decreases epithelial Na^+ channel (ENaC) expression and BP.[43] However, the expression of the insulin receptor in *Snx1*$^{-/-}$ mice is similar to that in wild-type littermates. The reduced LEPR expression in *Snx1*$^{-/-}$ mice may also not explain the lack of salt sensitivity of *Snx1*$^{-/-}$ mice because *Lepr*-deficient rats are salt-sensitive.[44]

4.2 | The phenotypes observed in *Snx1*$^{-/-}$ mice may translate to human pathology

There is a paucity of information relating SNX1 dysfunction or loss with any specific disease condition. One report showed that SNX1 expression is reduced in 75% of human colon cancers and that this may play a role in the development and aggressiveness of human

colon carcinogenesis.[45] Interestingly, hypertension is associated with a slight increase in cancer risk.[46] Moreover, microRNA-95 (miR-95), which inhibits the expression of SNX1, is increased in colon cancer tissues and induces cellular proliferation in non-small cell lung cancer.[47] To determine the clinical relevance of SNX1 loss in the pathogenesis of human hypertension, we profiled the basal expression of endogenous SNX1 in hRPTCs obtained from NT and HT Euro-American males. We have reported that an acute loss of SNX1 perturbs D_5R trafficking and signaling in hRPTCs.[11] We, now, report that hRPTCs from HT subjects have an inherent deficiency of SNX1, which results in impaired D_5R trafficking and activity and increased oxidative stress. Considering the modest expression of SNX1 in the kidney,[12] any reduction in SNX1 abundance in hRPTCs may conceivably become critical to the impairment of D_5R-mediated Na^+ excretion and negative regulation of ROS, resulting in hypertension. Moreover, we also found that the specific NOX inhibitor VAS2870 prevented the increase in ROS production and normalized the impaired fenoldopam-mediated inhibition of NKA activity in *SNX1*-specific siRNA-treated hRPTCs. These results indicate that NOXs are involved in these effects.

4.3 | Some *SNX1* SNPs may be relevant to human health

The human *SNX1* gene contains SNPs in both coding and noncoding regions that may be relevant to human pathology. Of the 12 *SNX1* SNPs that were interrogated for their effect on the BP response to antihypertensive therapy in the patients of the PEAR study, variant alleles of rs11635627 and rs11854249 were associated with antihypertensive effect of HCTZ monotherapy among African-American HT patients; variant allele rs12591927 trended toward a significant antihypertensive response to HCTZ monotherapy. These results suggest their relevance in the pharmacogenomics of hypertension. These three SNPs are not found within the coding region of the *SNX1* gene but rather map at the 3′ downstream sequence. While the exact mechanisms on how these SNPs affect SNX1 expression and/or function have yet to be described, variations at the 3′ UTRs may affect the recognition sites for miRNAs and RNA-binding proteins resulting in dysregulated gene expression. Variations at the 3′ downstream region may affect enhancer elements, chromatin regulators, and nucleosome positioning signals that may disrupt normal gene expression, chromatin formation, and nucleosome integrity. We assessed these noncoding variants using HaploReg [48] and found a 10- to 13-fold enrichment in all enhancers in epithelial cells and B-lymphocytes and a 28- to 64-fold enrichment in DNase hypersensitivity in B-lymphocytes, HepG2 cells, epidermal melanocytes, and hippocampal astrocytes. Ultimately, the presence of the minor allele of these SNPs may impair *SNX1* expression or be linked to SNPs that impair SNX1 function.

The association of the *SNX1* SNPs with a reduction in systolic BP with HCTZ could be related to D_5R function because HCTZ inhibits NCC which is expressed in the distal convoluted tubule of the nephron, a nephron segment where D_5R is also abundantly expressed.[7] $Snx1^{-/-}$ mice, compared with wild-type littermates, have increased renal NCC expression, similar to $Drd5^{-/-}$ mice which exhibit increased renal expression of NCC, NKCC2, and the α and γ subunits of ENaC.[7] Chronic AT_1R blockade with losartan does not normalize the expression of these Na^+ transporters, suggesting a direct inhibitory effect of D_5R. The hypertension in $Drd5^{-/-}$ mice is, in part, due to increased sympathetic tone that

may involve the adrenal catecholamine production.[49] However, none of the SNX1 variants is associated with BP response to the β_1 adrenoceptor antagonist atenolol. The interaction of the D_1-like receptors with the β_1-adrenergic receptor has not been described, although both D_1R and D_5R interact with the α_{1A}-adrenergic receptor in hRPTCs and kidneys of mice.[31]

In a separate population, our preliminary data indicate that the *SNX1*^{D466N} polymorphism (rs1802376) in exon 15 has significant genotypic and allelic association with essential hypertension in an Euro-American population ($P < .05$, 143 HTs, 207 pre-hypertensives, and 152 NT controls). The *SNX1* gene locus is also strongly associated with coronary artery disease.[50] These observations indicate a realistic translation of SNX1 loss to the development of hypertension or other types of cardiovascular disease in humans.

Taken together, our data underscore the crucial role of SNX1 in renal D_5R trafficking, signaling, and activity in the regulation of renal Na^+ handling and BP. In the absence of SNX1, D_5R function is impaired, resulting in its inability to inhibit the activity and expression of renal NKA, renal expression of NHE3, NCC, NOX1, NOX2, $p47^{phox}$, and promote the degradation of AT_1R. These result in increased oxidative stress, impaired pressure-natriuresis, and high BP that is unresponsive to changes in dietary salt intake.

5 | PERSPECTIVES

We have reported that the acute renal-restricted depletion of SNX1 results in blunted natriuretic response and high BP in mice due to impaired D_5R activity. The present study reinforces the role of SNX1 in hypertension and elucidates the molecular mechanisms, for example, oxidative/nitrosative stress, involved in the congenital absence of SNX1 in mice and hRPTCs. In addition, we also show an association of several *SNX1* SNPs with antihypertensive response to HCTZ monotherapy among HT African-Americans in the PEAR study. The results illustrate a new paradigm in the development of hypertension and imply that the trafficking protein SNX1 may be a crucial determinant for hypertension and responder to antihypertensive therapy. Studies using inducible kidney-specific *Snx1* knockout rodents and their corresponding renal epithelial cell lines are needed to corroborate the findings in our present study. An omics approach is also needed to compare and contrast the salient processes (genetics, genomics, proteomics, and metabolomics) that differentiate the kidneys of *Snx1* knockout from wild-type mice.

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Abbreviations:

AT_1R	angiotensin II type 1 receptor
BP	blood pressure
D_5R	dopamine D_5 receptor
GPCRs	G protein-coupled receptors
HCTZ	hydrochlorothiazide
HO-1	heme oxygenase 1
hRPTCs	human renal proximal tubule cells
NCC	thiazide-sensitive NaCl cotransporter
NHE3	Na^+-hydrogen exchanger 3
NKA	Na^+-K^+-ATPase
NKCC2	Na^+-potassium 2 chloride cotransporter
NOX	NADPH oxidase
PEAR study	Pharmacogenomic Evaluation of Antihypertensive Responses study
PON2	paraoxonase 2
ROS	reactive oxygen species
SNX1	sorting nexin 1

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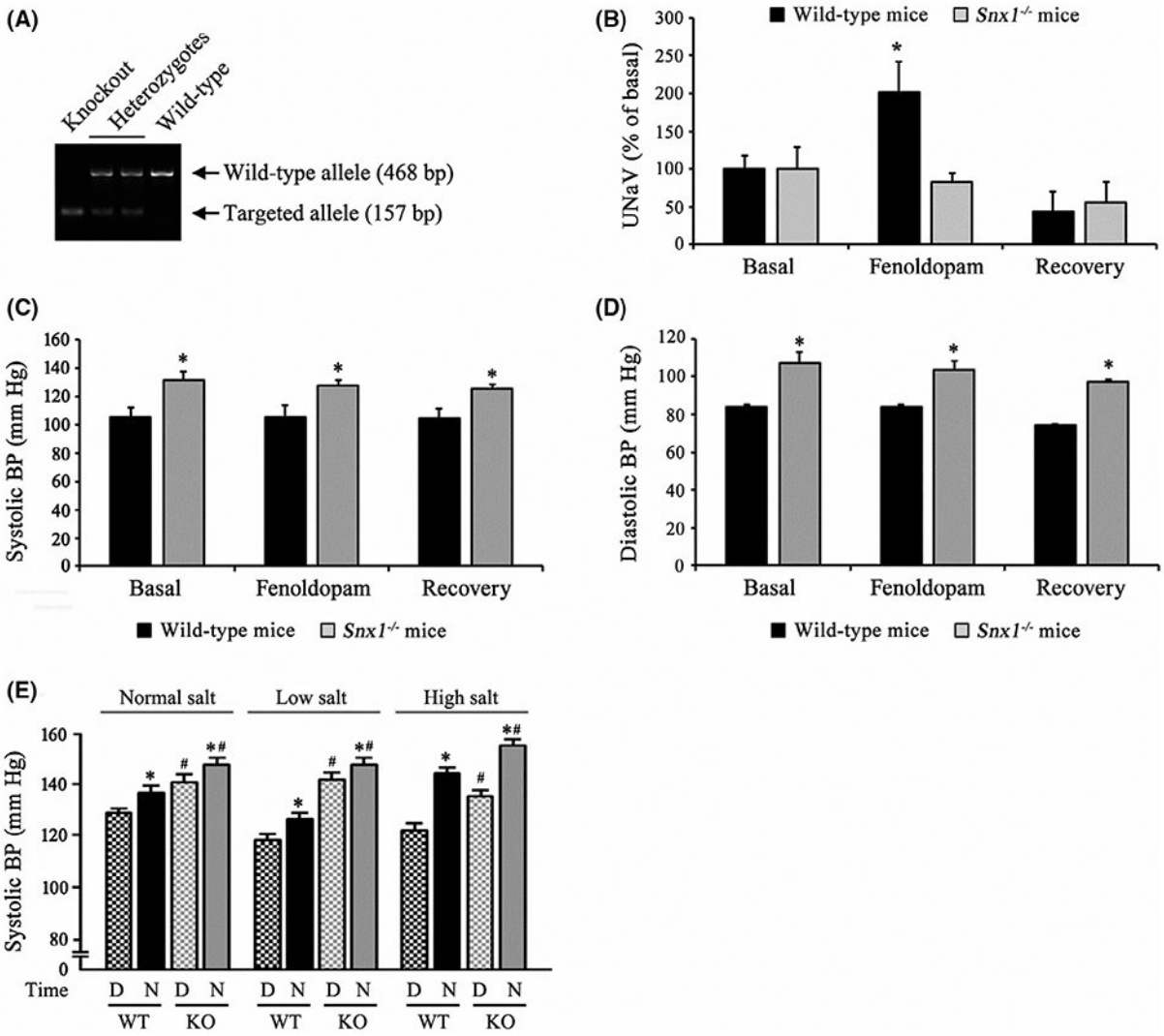

FIGURE 1.

Blood pressure and Na^+ excretion of *Snx1*$^{-/-}$ mice. Adult littermates were genotyped via PCR, according to published protocol.[15] A, The mice were anesthetized with pentobarbital (50 mg/kg body weight, intraperitoneal) prior to experimentation. Normal saline equivalent to 5% of the body weight was infused intravenously for 30 min. Urine samples (B) were collected and BPs (C and D), measured from the aorta, via the femoral artery, were recorded every hour starting with baseline followed by D_1R/D_5R agonist fenoldopam (2 μg/kg/min, intravenous, for 1 h) treatment, and then, a recovery period (60 min/period). Urinary Na^+ was calculated as urine volume (μl/min) x Na^+ (mEq/L). *$P < .05$, vs others, One-way ANOVA and Holm-Sidak post hoc test (B), or vs WT littermates (C and D), Student's *t* test, n = 8-9/group. E, Another group of *Snx1*$^{-/-}$ (KO) mice and wild-type (WT) littermates were fitted with telemeters inserted into the carotid artery and threaded into the aorta to monitor the daytime (D) and nighttime (N) BP. These mice were fed consecutively, normal (0.6% NaCl), low (<0.02% NaCl), and high (6%) salt diet for one wk. *$P < .05$, vs daytime BP, $^{\#}P < .05$, vs WT littermates, same time of day, one-way ANOVA and Holm-Sidak post hoc test, n = 8-9/group. Numerical data are expressed as mean ± SEM

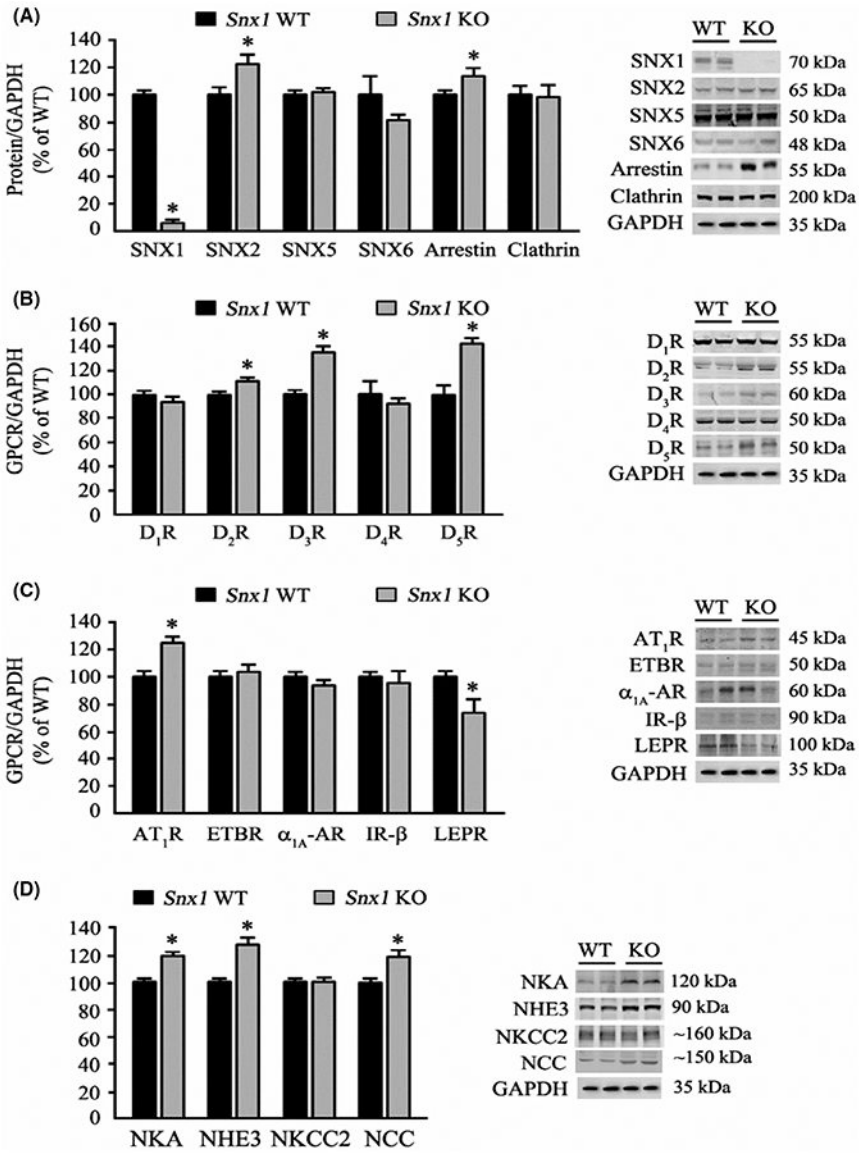

FIGURE 2.

Expression profiles of select proteins in the kidney of *Snx1*$^{-/-}$ mice. Protein expression was evaluated via immunoblotting for proteins involved in receptor endocytosis and trafficking (A), dopamine receptors (B), other receptors of interest (C), and Na^+ exchanger, transporters/pump (D) in the kidney of *Snx1*$^{-/-}$ (knockout, KO) mice and wild-type (WT) littermates. GAPDH was used for normalization. Representative immunoblots are shown. Numerical data are expressed as mean ± SEM. **P* < .05, vs *Snx1* WT, Student's *t* test, n = 9/group

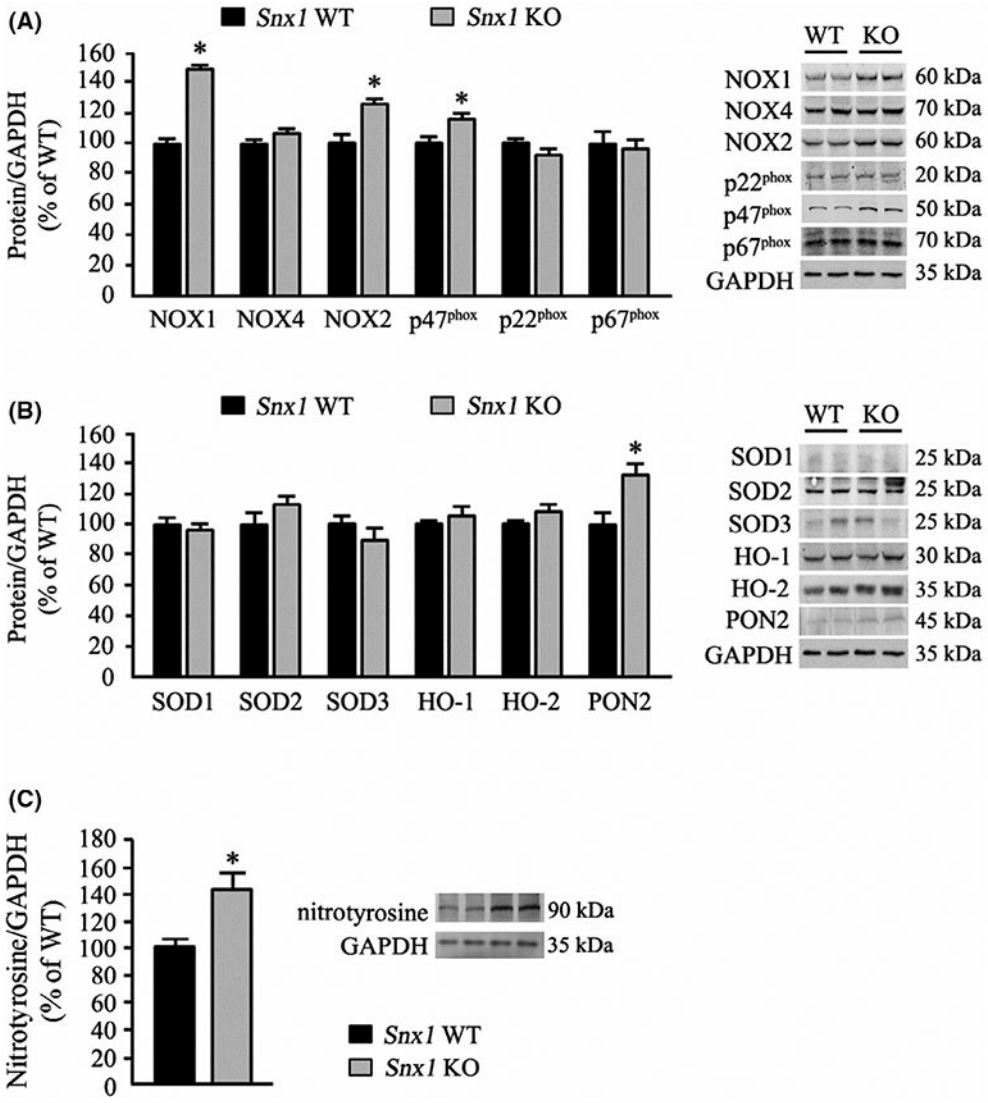

FIGURE 3.

Expression profiles of NADPH oxidase subunits, antioxidant enzymes, and nitrotyrosine in *Snx1*$^{-/-}$ mice. Protein expression was determined for the components of NADPH oxidase (NOX) (A), select enzymes with antioxidant activity (B), and nitrotyrosine protein (C) in the kidney of *Snx1*$^{-/-}$ (knockout, KO) mice and wild-type (WT) littermates. GAPDH was used for normalization. Representative immunoblots are shown. Numerical data are expressed as mean ± SEM. **P* < .05, vs *Snx1* WT, Student's *t* test, n = 9/group

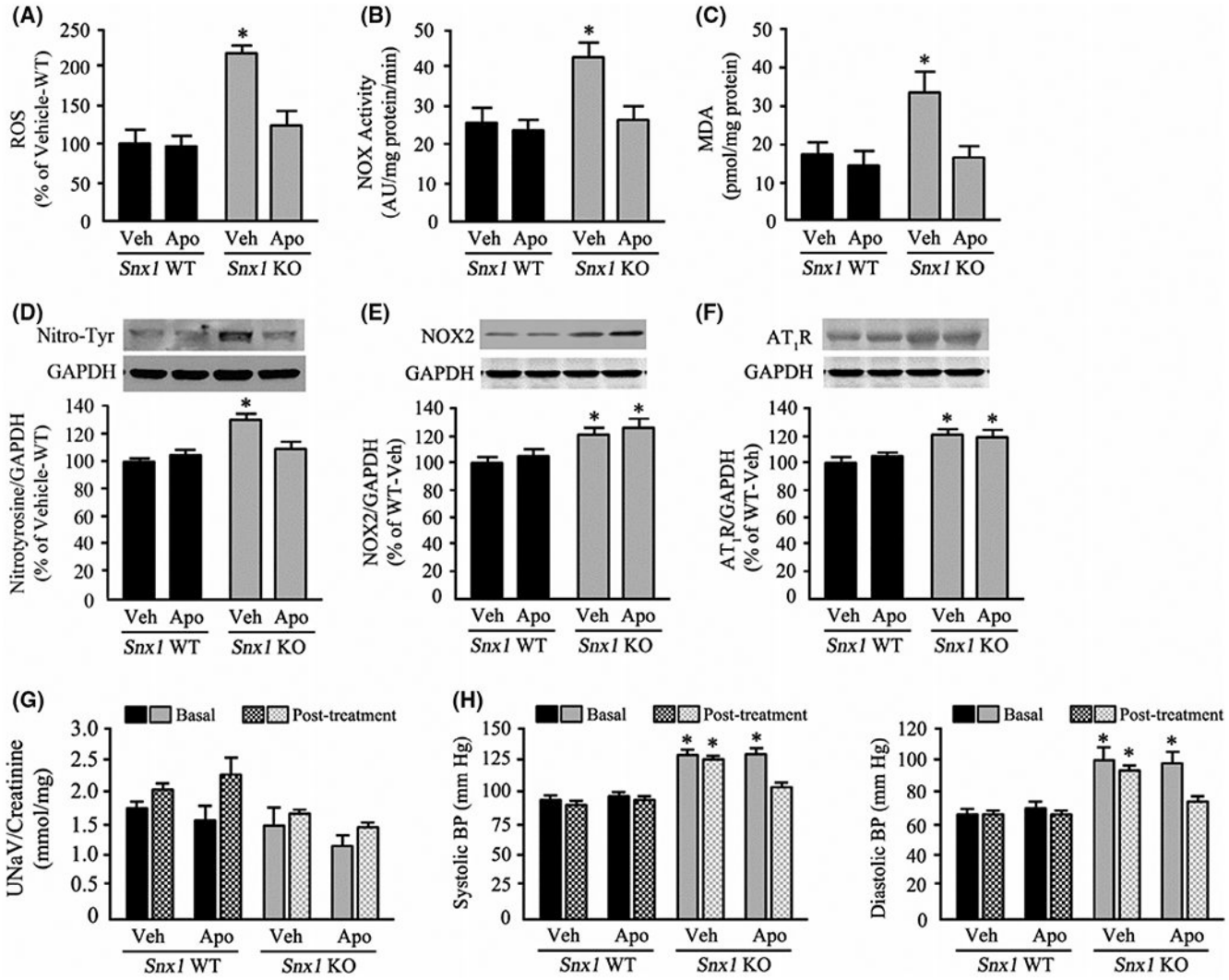

FIGURE 4.

Effects of apocynin on renal oxidative stress, lipid peroxidation, and nitrosative stress, AT_1R, and BP in *Snx1*$^{-/-}$ mice. *Snx1*$^{-/-}$ (knockout, KO) mice and wild-type (WT) littermates were treated with apocynin (Apo; 3 mg/kg/day) or vehicle (Veh) via subcutaneous infusion with osmotic minipump for 10 days. Reactive oxygen species (ROS) levels (A), NADPH oxidase (NOX) activity (B), malonyldialdehyde (MDA) (marker of lipid peroxidation) levels (C), and ~ 90-kDa nitrotyrosine (marker of reactive nitrogen species) levels (D) in total kidney homogenates. Expression profiles of renal NOX2 (E) and AT_1R (F) normalized for GAPDH. G: Urinary Na^+ corrected for creatinine from 24-h urine voided by the mice housed in metabolic cages before and after apocynin treatment. H, Systolic and diastolic BP measured under pentobarbital (50 mg/kg body weight, intraperitoneal) anesthesia prior to the start of apocynin treatment and after the 10-day apocynin treatment period. Numerical data are expressed as mean ± SEM. *$P < .05$, vs others, one-way ANOVA and Holm-Sidak post hoc test, n = 8/group

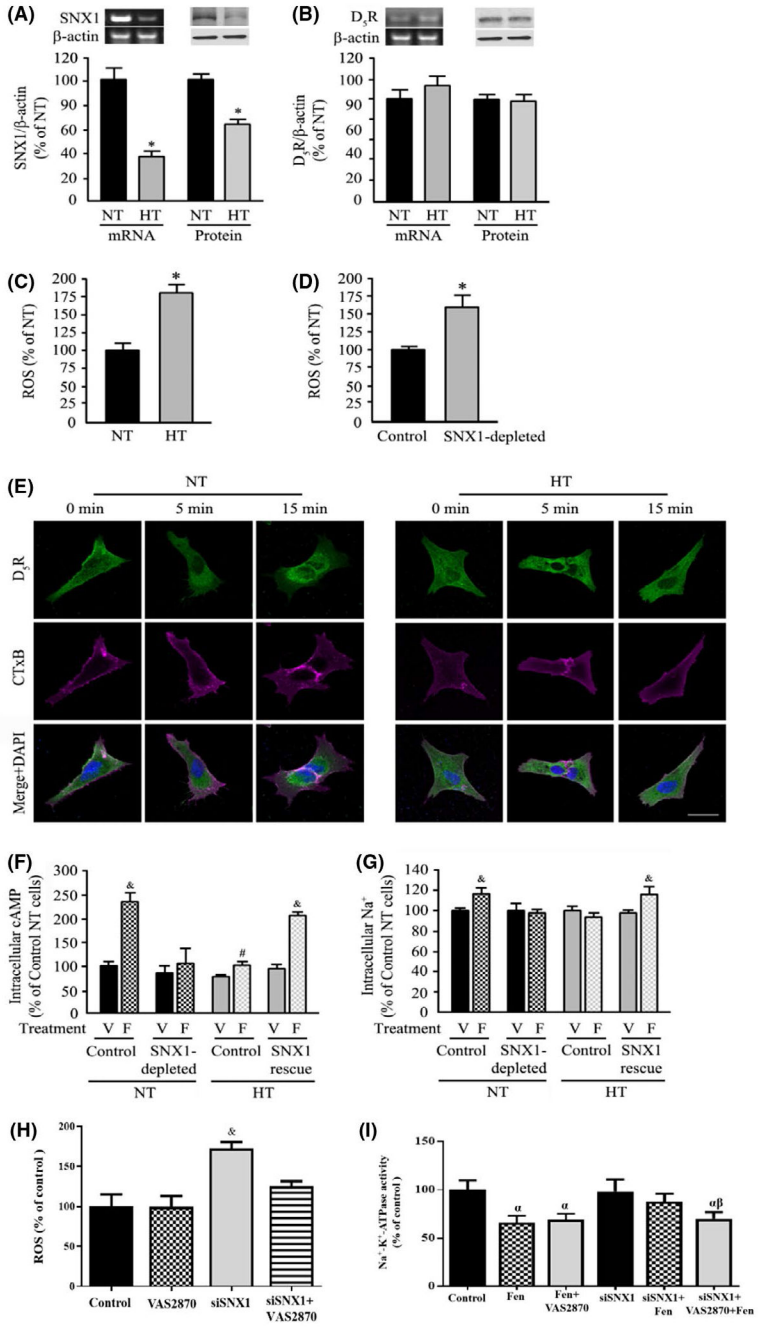

FIGURE 5.

Comparison of renal proximal tubule cells from normotensive and hypertensive Euro-American males. A, Expression of SNX1 normalized for β-actin in immortalized hRPTCs obtained from normotensive (NT) and hypertensive (HT) Euro-American males. B, D_5R normalized for β-actin in immortalized hRPTCs obtained from normotensive (NT) and hypertensive (HT) Euro-American males. C and D, Reactive oxygen species (ROS) in hRPTCs from NT and HT subjects and hRPTCs from NT subjects in the basal state (C) or transfected with non-silencing siRNA (Control) or *SNX1*-specific siRNA (SNX1-depleted)

(D). E, D_5R (pseudocolored green) internalization in response to fenoldopam (1 μM, at the indicated time points) in hRPTCS from NT and HT subjects. The plasma membrane (pseudocolored magenta) was labeled with cholera toxin subunit B (CTxB) tagged with Alexa Fluor 647. DAPI was used to visualize the nuclei (pseudocolored blue). A 600x magnification, scale bar = 10 μm. F and G, Effect of fenoldopam (1 μM/15 min) on intracellular cAMP and Na^+ in hRPTCS from NT subjects depleted of SNX1 via *SNX1*-siRNA and hRPTCs from HT subjects "rescued" by the heterologous overexpression of SNX1. H, ROS production in *SNX1*-specific siRNA (siSNX1)-transfected hRPTCs with or without VAS2870 (5 μM), a specific NOX inhibitor. I, Effect of VAS2870 (5 μM) on fenoldopam (Fen, 1 μM/15 min)-induced inhibition of Na^+-K^+-ATPase activity in control and *SNX1*-specific siRNA (siSNX1)-transfected hRPTCs. $^*P < .05$, vs NT, Student's *t* test, n = 5/group. $^{\&}P < .05$, vs others, one-way ANOVA and Holm-Sidak post hoc test, n = 5/group. $^{\#}P < .05$, vs V Control, HT (intracellular cAMP), Student's *t* test, n = 5/group. $^{\alpha}P < .05$, vs Control, one-way ANOVA and Holm-Sidak post hoc test, n = 4-5/group. $^{\beta}P < .05$, vs Fen + siSNX1, Student's *t* test, n = 4-5/group

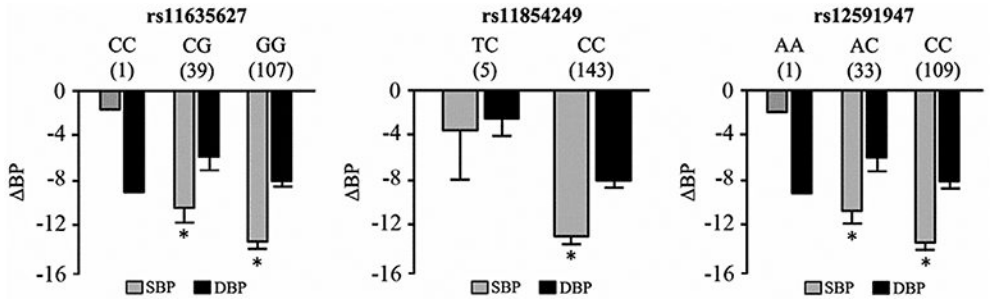

FIGURE 6.

BP response to HCTZ monotherapy by *SNX1* genotype in African-Americans. Changes in SBP and DBP (mm Hg) in subjects in response to HCTZ monotherapy according to *SNX1* SNPs. The genotypes and corresponding number of subjects are indicated in parentheses. **P* = .013 for SBP, rs11635627, **P* = .009 for SBP, rs11854249, and **P* = .019 for SBP, rs12591947. Analysis was performed using PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) according to an additive model

TABLE 1

Minor allele frequency (MAF), betas, and *P* values for the change in systolic (SBP) and diastolic (DBP) BP in response to HCTZ treatment for each *SNX1* SNP in the PEAR study

		Euro-Americans					African-Americans				
SNP	**Alleles**	**MAF**	**Beta (SBP)**	***P* (ΔSBP)**	**Beta (DBP)**	***P* (ΔDBP)**	**MAF**	**Beta (SBP)**	***P* (ΔSBP)**	**Beta (DBP)**	***P* (ΔDBP)**
rs332258	C/G	0.23	−0.9	.28	0.2	.70	0.34	−0.1	.91	0.1	.53
rs11629723	T/C	0.29	0.4	.58	−0.5	.32	0.12	2.2	.13	0.03	.33
rs2290117	C/A	0.23	−0.9	.27	0.2	.71	0.43	−0.2	.81	0.08	.55
rs3816385	G/A	0.23	−0.9	.27	0.2	.71	0.44	−0.9	.33	−0.2	.34
rs34257057	T/G	0.06	1.4	.35	1.4	.16	0.008	−8.1	.30	−1.7	.24
SNP15-62215612	A/G	0.02	−1.4	.52	0.2	.92	0.003	−0.6	.92	NA	.47
rs3848144	T/A	0.00	NA	NA	NA	NA	0.09	−0.4	.83	−0.3	.39
rs749504	A/G	0.29	0.4	.61	−0.5	.30	0.12	2.2	.13	0.03	.33
rs7176480	T/G	0.001	0.6	.93	−1.6	.73	0.28	0.2	.82	0.2	.69
rs12591947	C/A	0.29	0.2	.75	−0.6	.22	0.13	3.3	.019	0.2	.12
rs11854249	T/C	0.00	NA	NA	NA	NA	0.02	9.1	.009	1.6	.046
rs11635627	G/C	0.29	0.2	.75	−0.6	.22	0.13	3.4	.013	0.2	.08

Note: Significant *P* values (<.01) are in red. $P < .01$ was considered significant given the linkage disequilibrium between the SNPs, for example, rs12591947 is in strong linkage disequilibrium ($r^2 = 0.99$) with rs11635627.