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INCREASED RENAL OXIDATIVE STRESS IN SALT-SENSITIVE HUMAN GRK4γ486V TRANSGENIC MICE

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Abstract

We tested the hypothesis that salt-sensitive hypertension is caused by renal oxidative stress by measuring the blood pressure and reactive oxygen species-related proteins in the kidneys of human G protein-coupled receptor kinase 4γ (hGRK4γ) 486V transgenic mice and non-transgenic (Non-T) littermates on normal and high salt diets. High salt diet increased the blood pressure, associated with impaired sodium excretion, in hGRK4γ486V mice. Renal expressions of NOX isoforms were similar in both strains on normal salt diet but NOX2 was decreased by high salt diet to a greater extent in Non-T than hGRK4γ486V mice. Renal HO-2, but not HO-1, protein was greater in hGRK4γ486V than Non-T mice on normal salt diet and normalized by high salt diet. On normal salt diet, renal CuZnSOD and ECSOD proteins were similar but renal MnSOD was lower in hGRK4γ486V than Non-T mice and remained low on high salt diet. High salt diet decreased renal CuZnSOD in hGRK4γ486V but not Non-T mice and decreased renal ECSOD to a greater extent in hGRK4γ486V than Non-T mice. Renal SOD activity, superoxide production, and NOS3 protein were similar in two strains on normal salt diet. However, high salt diet decreased SOD activity and NOS3 protein and increased superoxide production in hGRK4γ486V mice but not in Non-T mice. High salt diet also increased urinary 8-isoprostane and 8-hydroxydeoxyguanosine to a greater

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AUTHOR DISCLOSURE STATEMENT

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

extent in hGRK4γ486V than Non-T mice. hGRK4γwild-type mice were normotensive and hGRK4γ142V mice were hypertensive but both were salt-resistant and in normal redox balance. Chronic tempol treatment partially prevented the salt-sensitivity of hGRK4γ486V mice. Thus, hGRK4γ486V causes salt-sensitive hypertension due, in part, to defective renal antioxidant mechanisms.

Keywords

hypertension; oxidative stress; kidney; SOD; NOS3; tempol

INTRODUCTION

Salt sensitivity is associated with increased production of reactive oxygen species (ROS), including superoxide and hydrogen peroxide, in some humans with hypertension and rodent models of hypertension (1–5). The activation of the renin-angiotensin-aldosterone system (RAAS) increases ROS production, in part via stimulation of NADPH oxidases (NOXs) (2, 3, 5, 6). The angiotensin type 1 receptor (AT_1R)-mediated increase in ROS production along the nephron also augments its ability to increase renal sodium transport (2, 4–7). Thus, the RAAS, including the AT_1R in the kidney, is critical in the overall positive regulation of fluid and electrolyte balance and blood pressure (BP) (1, 2, 4, 6–8).

Oxidative stress is caused not only by an increase in ROS production but also by a decrease in the scavenging of ROS. For example, angiotensin II increases ROS production by stimulating NOX activity and by decreasing the dismutation of superoxide into O_2 and H_2O_2, via inhibition of superoxide dismutases (SOD) (2, 3, 5). Angiotensin II can also decrease the activity of MnSOD by increasing its nitration (9, 10). Decreased renal expression of SOD may also be involved in the hypertension associated with the dysfunction or decreased expression of the dopamine receptors D_1R, D_2R, and D_5R (11–13).

Heme oxygenases (HO) are important in the anti-oxidative function of dopamine receptors. They degrade heme, preventing it from forming ROS; HO also produces CO and bilirubin that can activate antioxidant genes (12, 13). The renal protein abundance of HO is decreased in mice with a genetic disruption of the dopamine receptors D_2R (12) and D_5R (13). Antioxidants, by counteracting prooxidants, keep the redox balance normal and consequently a normal BP (2, 3, 5, 7, 11–13), or at least limit the increase in positive sodium balance and BP induced by angiotensin II (6, 7, 14). The increase in ROS caused by angiotensin II can be mitigated by an increase in HO-1 expression (15–17).

The 486V variant of the human G protein-coupled receptor kinase type 4 (hGRK4), either by itself or in conjunction with other hGRK4 variants (65L and 142V), is associated with salt-sensitive hypertension in Japanese and Italians (18, 19). GRK4 in the kidney regulates both angiotensin and dopamine receptors and is influenced by salt intake (1, 5, 20, 21). Because high salt intake also increases ROS production (5, 22, 23), it may exaggerate the defects in antioxidant mechanisms related to a GRK4-induced abnormal interaction between angiotensin and dopamine receptors to cause salt- sensitive hypertension (1, 5, 20, 21, 24, 25). The salt sensitivity of the spontaneously hypertensive rat (SHR) is associated (26) with

an aberrant interaction between angiotensin and dopamine receptors in the kidney (24). In the SHR, the intrarenal cortical infusion of GRK4 or AT_1R antisense oligodeoxynucleotides decreases BP and increases natriuresis. However, the combined intrarenal cortical infusion of GRK4 and AT_1R antisense oligodeoxynucleotides in the SHR causes a greater natriuresis and amelioration of hypertension than by either GRK4 or AT_1R antisense oligodeoxynucleotides alone (24).

The role renal oxidative stress and salt sensitivity in GRK4-mediated hypertension has not been evaluated. Moreover, the renal mechanisms involved in the salt sensitivity caused by hGRK4γ486V have not been determined. The current experiments were designed to test the hypothesis that increased renal oxidative stress causes salt-sensitive hypertension in hGRK4γ486V transgenic mice. The results were also compared with those observed in hGRK4γ142V and wild-type (hGRK4γWT) transgenic mice.

MATERIALS AND METHODS

Human GRK4γ486V transgenic and Non-T mice

Mice carrying hGRK4γ486V were generated at the University of Michigan Transgenic Core Facility, using a protocol similar to that used to generate hGRK4γ142V transgenic mice (27–29). The mice were crossbred with SJL and C67Bl6/J mice. The genetic background of mice used in the current study is 30% SJL and 70% C67Bl6/J.

Human GRK4γ142V and hGRK4γWT transgenic mice and Non-T littermates

Mice carrying hGRK4γ142V were also generated at the University of Michigan Transgenic Core Facility (27–29) and bred in our laboratory. These mice are in more than 98% C67Bl6/J genetic background. All mice were genotyped at 21 days of age.

Salt diets, metabolic studies, BP measurements, and tempol treatment

Male and female mice (4–5 months old, n=10–11/strain) were randomly divided into 2 groups to determine the effect of high salt intake on BP and renal protein expression of genes of interest. Mice were fed regular mouse chow with 0.8–0.9% NaCl, designated as normal salt diet, and 6% NaCl, designated as high salt diet. The mice, with free access to food and water, were fed these diets for 3 weeks. Their room was on a 12:12 light cycle and the temperature maintained between 20° and 26°C. Urine samples, for sodium and creatinine measurements, were collected in metabolic cages for 24 hr on the 21st day of the diet. After the samples were collected, the mice were anesthetized with ketamine/xylazine (0.1 mg/100 kg, intraperitoneal injection) and arterial BP was measured (CardioMaxII, Columbus Instrument) in the aorta via a catheter inserted into the femoral artery (12, 27–31).

In order to determine the role of ROS and nitric oxide in the BP phenotype of these mice, an additional set of mice were fed high salt diet for 3 weeks; tempol was added in drinking water (1 mmol/L) in the last 2 weeks of the high salt diet. BP was measured before (day 7) and two weeks (day 21) after the addition of tempol in the drinking water.

All mouse studies were conducted in accordance with NIH guidelines for the ethical treatment and handling of animals in research and approved by the Institutional Animal Care and Use Committee of Georgetown University and the University of Maryland, Baltimore.

Semi-quantitative immunoblotting

After the mice were euthanized with an overdose of pentobarbital (100/mg/kg) administered by rapid intravenous injection, the kidneys were immediately removed and frozen, until use (12, 27–31). The thawed kidneys were homogenized in 250 mM sucrose, 10 mM triethanolamine, with protease inhibitors. The proteins in the samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were probed with the indicated primary antibodies and then exposed to horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL). The polyclonal antibodies against the proteins of interest were: NOX1 (SC5821, Santa Cruz, Dallas, Texas); NOX2 (07–024, Upstate, Billerica, MA); CuZnSOD, MnSOD (SOD101, SOD111, Stressgen, Ann Arbor, MI); ECSOD, nitrotyrosine (Ab83108, 42789, Abcam, Cambridge, MA); NOS1/NOS2 (SC648, SC651, Santa Cruz); NOS3 (610296, BD Biosciences, San Jose, CA); HO-1(SC10789, Santa Cruz); HO-2 (ADI-OSA-200, Enzo, Farmingdale, NY), and actin (A2066, Sigma, St. Louis, MO). The use of affinity-purified polyclonal rabbit antibodies against NOX4 generated in our laboratory was reported previously (27). The chemiluminescent signals were detected using Amersham Hyperfilm (GE, Little Chalfont, UK) and the band densities were quantified by the NIH Image J program and normalized with the corresponding actin bands. IRDye® 800CW and IRDye® 680RD secondary antibodies were used in some blots and the signals were detected and analyzed using the Odyssey imaging system (LI-COR, Lincoln, NE).

ROS production and NOX activity in the kidney

The kidney homogenates (40 μg of protein from each sample, total volume 100 μl/well) were incubated with 10 μM lucigenin (Sigma), a chemiluminescent probe, that measures total ROS, including superoxide and hydrogen peroxide (32, 33), at 37°C for 10 min before chemiluminescence was measured by Centro LB 960 with automatic injection system (Dana Scientific, Dresher, PA). NOX-derived superoxide was also measured with lucigenin, similar to total ROS measurement but in the presence of NADPH (100 μM). The specificity of the NADPH-dependent superoxide production was verified using diphenyleneiodonium (Sigma). NOX production and NOX activity were corrected for total protein, quantified with a BCA kit (Pierce, Rockford, IL).

Total SOD activity in kidney

Total SOD activity was measured in kidney homogenates with Colorimetrc EpiQuik Superoxide Dismutase Activity/Inhibition Assay Kit (Epigentek, Farmingdale, NY). The activity was corrected for total protein concentration measured with a BCA kit (Pierce).

Urine and serum measurements

The concentration of nitrite/nitrate in urine was measured with a two-step Colorimetric Assay Kit (780001, Cayman, Ann Arbor, MI) for NO metabolite detection. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess reagent which converts nitrite into a deep purple azo compound and measured at 540 nm (Fisher, Pittsburgh, PA). Urinary nitrite/nitrate was corrected for creatinine concentration. Urine 8-isoprostane (516351, Cayman), 8-hydroxydeoxyguanosine (8-OHdG, 50154038, Fisher Scientific, Waltham, MA), aldosterone (50946889, Fisher), and serum renin (501044785, Fisher) were measured with ELISA kits.

H&E and immunofluorescence staining

Additional GRK4γ486V transgenic mice and non-T littermates were fed normal and high salt diet for 3 weeks (n=2 per group). After euthanasia, the kidneys were perfused with phosphate-buffered saline and fixed with Histochoice (AMRESCO LLC, Solon, OH). Two-μm sections of paraffin-embedded kidney sections were cut and mounted onto slides. H&E staining was used to evaluate kidney morphology under a DMLB microscope (Leica Microsystems Inc, Buffalo Grove, IL). For immunofluorescence studies, the slides were pretreated with non-fluorescent goat-anti-mouse IgG and goat serum to block non-specific staining. The immunofluorescence signals of ECSOD (rabbit polyclonal antibody) and NOS3 (mouse monoclonal antibody) were visualized with secondary donkey anti-rabbit (conjugated with Alexa Fluor® 488, Invitrogen, Eugene, OR) and donkey anti-mouse (conjugated with Alexa Fluor® 555, Invitrogen) antibodies. In addition, mouse monoclonal antibody against 8-OHdG (Ab48508, Abcam, cloneN45.1) and donkey anti-mouse secondary antibody (conjugated with Alexa Fluor® 488, Invitrogen) were used. Anti-fade mounting medium (Molecular Probes, Eugene, OR) was used together with 4,6-diamidino-2-phenylindole (DAPI, Life Technologies, Grand Island, NY) to stain the nucleus and wheat germ agglutinin (WGA, conjugated with Alexa Fluor® 647, Life Technologies) to stain the plasma membrane. The immunofluorescence was observed with a confocal microscope (LSM5) and the images analyzed with Zen 2011 software (Carl Zeiss Microscopy, Jena, Germany).

Statistical analysis

Immunoblotting band density, corrected for actin as housekeeping protein, was expressed as mean ± SE and shown as percent of non-T mice on normal salt diet. One-way factorial ANOVA and Holm-Sidak post-hoc test were used for multi-group (>2) comparison. Student's t-test was used for two- group comparison. $P<0.05$ was considered significant.

RESULTS

Effect of hGRK4γ486Vtransgene on pressure-natriuresis

Systolic BP (SBP), measured under anesthesia, was similar in hGRK4γ486V transgenic mice and nontransgenic (Non-T) littermates on normal salt diet. However, high salt diet increased SBP, also measured under pentobarbital anesthesia, in hGRK4γ486V but not Non-T mice (Figure 1A), indicating that hGRK4γ486V transgenic mice are salt-sensitive. The

salt sensitivity of the hGRK4γ486V transgenic mice was confirmed in additional non-anesthetized, conscious mice whose BPs were monitored by telemetry (unpublished data, Z. Wang). Urine sodium excretion was similar in the two mouse strains on normal salt diet and increased by high salt diet in both mouse strains. However, the plot of urine sodium excretion against SBP, a measure of pressure-natriuresis, was shifted to the right in hGRK4γ486V transgenic mice, indicating that a higher BP is needed to excrete the same amount of sodium (Figure 1B). Table 1 shows the physiological data in the mice before and after consumption of normal or high salt diet. The mice had similar body weights, regardless of diets. Water and food intakes, urine volume, urinary sodium excretion (absolute or corrected by urine creatinine), and urine chloride excretion were increased while urinary potassium excretion in Non-T and urine creatinine concentration in both mouse strains were decreased by HS diet. The serum concentrations of sodium, potassium, and chloride were similar except for a small increase in serum potassium in hGRK4γ486V mice on HS diet.

Effect of hGRK4γ486V transgene on renal NOX protein abundance and activity

The renal protein abundance of NOX1, NOX2, and NOX4 was similar in the two mouse strains on normal salt diet. High salt diet did not affect renal NOX1 or NOX4 expression in either mouse strain. However, high salt diet decreased renal NOX2 protein expression in both mouse strains but to a greater extent in Non-T than hGRK4γ486V transgenic mice (Figures 2A&2B). Total renal NOX activity was similar in hGRK4γ486V transgenic mice and Non-T littermates on normal salt diet. High salt diet caused a slight increase in NOX activity in both mouse strains, although the increase was significant only in hGRK4γ486V transgenic mice (Figure 2C).

Effect of hGRK4γ486V transgene on renal SOD protein abundance and activity

On normal salt diet, the renal protein abundance of CuZnSOD and ECSOD was similar in the two mouse strains but MnSOD was lower in hGRK4γ486V transgenic than Non-T mice. However, high salt diet decreased renal CuZnSOD in hGRK4γ486V transgenic but not Non-T mice. High salt diet did not affect renal MnSOD in either mouse strain such that its expression remained lower in hGRK4γ486V transgenic than Non-T mice. By contrast, high salt diet decreased renal ECSOD to a greater extent in hGRK4γ486V transgenic than Non-T mice (Figures 3A & 3B). Total renal SOD activity was similar in the two mouse strains on normal salt diet but was decreased by high salt diet only in the hGRK4γ486V transgenic mice (Figure 3C).

Effect of hGRK4γ486V transgene on renal HO protein abundance

On normal salt diet, renal HO-1 protein was similar in the two mouse strains whereas HO-2 was greater in hGRK4γ486V than Non-T mice. High salt diet decreased renal HO-1 protein in both mouse strains, but reached significance only in Non-T mice. By contrast high salt diet decreased HO-2 in hGRK4γ486V mice to a level similar to that found in Non-T mice, which was not affected by the increase in sodium intake (Figures 4A & 4B).

Effect of hGRK4γ486V transgene on renal NOS protein abundance and urinary nitrite/nitrate excretion

The renal protein abundance of NOS1, NOS2, and NOS3 was similar in the two mouse strains. High salt diet did not affect renal NOS1 or NOS2 expression but decreased NOS3 expression only in hGRK4γ486V transgenic mice (Figures 5A & 5B). Urine nitrite/nitrate concentration tended to be higher in hGRK4γ486V transgenic than Non-T mice on normal salt diet. The high salt diet decreased urine nitrite/nitrate concentration in both mouse strains but reached significance only in hGRK4γ486V transgenic mice (Figure 5C).

Effect of hGRK4γ486V transgene on total renal oxidative markers

The level of oxidative stress in kidney was determined by renal nitrotyrosine protein abundance, ROS production, and urinary 8-isoprostane excretion. The total nitrotyrosine abundance in whole kidney homogenates was similar in the two mouse strains on normal salt diet and was similarly increased by high salt diet (Figures 6A & 6B). The similar increase in renal nitrotyrosine may not be sufficient to exclude the presence of increased oxidative stress in hGRK4γ486V mice even though nitrotyrosine reflects total production of peroxynitrite, a highly reactive oxygen species formed by the interaction of superoxide and NO (34). Indeed, while renal ROS production was similar in the two mouse strains on normal salt diet, high salt diet increased ROS production only in hGRK4γ486V mice (Figure 6C). In both mouse strains, urinary 8-isoprostane excretion was similar on normal salt diet and increased by high salt diet but the increase was greater in hGRK4γ486V than Non-T mice (Figure 6D). These results suggest that total oxidative stress in the kidney is increased by high salt diet in hGRK4γ486V transgenic mice.

Role of RAAS and ROS in the salt sensitivity of hGRK4γ486V transgenic mice

Serum renin concentration was similar in the two mouse strains on normal salt diet but was decreased by high salt diet only in Non-T mice (Figure 7A). Urine aldosterone concentration was similar in both mouse strains regardless of diet (not shown). Urine 8-OHdG was also similar in the two mouse strains on normal salt diet but was increased to a greater extent in hGRK4γ486V than Non-T mice by high salt diet. Tempol not only normalized urine 8-OHdG (Figure 7B) but also partially ameliorated the high salt-induced increase in BP in hGRK4γ486V transgenic mice (Figure 7C).

Effect of hGRK4γ142V and hGRK4γWT transgenes on salt sensitivity

The normal SBP of hGRK4γWT mice and high SBP of hGRK4γ142V mice on normal salt diet were not affected by high salt diet (Figure 7D). However, high salt diet slightly increased the SBP of their Non-T littermates, probably because these mice are >98% C57Bl6/J background; C57Bl/6J mice are salt-sensitive (35, 36). The presence of the 30% SJL/J salt-resistant (30) background of the Non-T littermates of hGRK4γ486V mice may have rendered them salt-resistant (Figure 1A). Although hGRK4γ142V mice are hypertensive even on normal salt diet, these mice have normal redox balance (27). Therefore, we studied the kidneys of the salt-resistant hGRK4γWT transgenic mice (>98% C57Bl/6J background) on high salt diet, as a control for the salt-sensitive C57Bl/6J (>98% C57Bl/6J background) Non-T littermates. Except for ECSOD, which was more abundant in

hGRK4γWT mice than their Non-T littermates on >98% C57BL/6J background without the transgenes, the renal protein abundance of CuZnSOD and MnSOD (Figures 8A), NOX1, NOX2, and NOX4 (Figures 8B), and NOS3 and nitrotyrosine (Figures 8C) were similar in hGRK4γWT mice and Non-T littermates. These results support the notion that increased renal oxidative stress may be a factor in the salt sensitivity in hGRK4γ486V transgenic mice. It is also possible that the increase in renal ECSOD with high salt diet in hGRK4γWT mice may have played a role in making the hGRK4γWT mice salt-resistant, in spite of their being in >98% C57Bl/6J background.

Effect of hGRK4γ486V transgene on kidney structure and immunostaining of ECSOD and NOS3

Another set of hGRK4γ486V mice and their Non-T littermates were fed normal or high salt diet. The H&E staining revealed normal kidney morphology in both groups (Figure 9A, upper panel). 8-OHdG staining was most intense in the kidney cortex of salt-loaded hGRK4γ486V transgenic mouse (Figure 9A, lower panel). ECSOD was observed in tubules and glomeruli. WGA staining showed that ECSOD was abundantly expressed in the apical membranes in some tubules. The staining for ECSOD was reduced in both mouse strains fed a high salt diet (Figure 9B), in agreement with the immunoblotting data (Figures 3A & 3B). Since the decrease in ECSOD expression was found in both strains fed the high salt diet, we optimized the immunostaining by adding more primary antibodies (ECSOD and NOS3) to the kidney sections of the mice treated with high salt diet in order to facilitate their comparison (Figure 9C). In both mouse strains, more ECSOD was expressed in renal tubules than glomeruli. NOS3 was similarly expressed in renal tubules and glomeruli. Colocalization of ECSOD and NOS3 was observed mainly in renal tubules. However, the staining of ECSOD and NOS3 was less in the mouse cortex from hGRK4γ486V than Non-T mice (Figure 9C), in agreement with the immunoblotting studies (Figures 3A, 3B, 5A, & 5B). Negative controls were those not treated with primary antibodies and imaged under the same specifications.

DISCUSSION

Increased renal oxidative stress is present in several rodent models of hypertension (1–3, 5–7, 9–17, 37). In the current study, we find that hGRK4γ486V transgenic mice are salt-sensitive based on the shift to the right of the pressure-natriuresis plot, in response to a high salt diet, relative to that observed on a normal salt diet. A blunted pressure-natriuresis response is also seen in salt-sensitive humans (4, 25, 37). Furthermore, the salt sensitivity of hGRK4γ486V transgenic mice is associated with renal oxidative stress. On normal salt diet, renal ROS production, measured by lucigenin, is similar in hGRK4γ486V mice and Non-T littermates. However, high salt diet increases renal ROS production only in hGRK4γ486V mice. Urinary 8-isoprostane is similar at baseline and increased by salt loading in both mouse strains. However, the increase in urinary 8-isoprostane is greater in hGRK4γ486V mice than Non-T littermates (Figure 6D). Renal oxidative stress may cause salt sensitivity by increasing the activity of renal sodium transporters, resulting in an increase in renal sodium and water reabsorption (1, 2, 5, 14). Indeed, high salt diet is associated with increased protein abundance of sodium hydrogen exchanger type 3 in the brush border

membrane of renal proximal tubules in hGRK4γ486V transgenic but not in Non-T mice (unpublished observations, X.Wang).

The genetic background of rodents influences ROS production that may or may not be associated with salt sensitivity. The salt sensitivity in Dahl salt-sensitive rats is related to increased ROS production (5, 23). However, increased oxidative stress *per se* may not always cause salt sensitivity. For example, SJL mice are salt-resistant (30), while C57Bl6/J mice are salt-sensitive (35, 36) but a high salt intake increases renal ROS production in SJL but not C57Bl6/J mice (30). Both hGRK4γ486V mice and Non-T littermates in the current study have more C57Bl6/J (70%) than SJL (30%) background, which may explain why high salt diet does not increase total renal ROS in the Non-T mice (Figure 6C). The 30% SJL background may have conferred salt resistance in the Non-T mice (30). The hGRK4γ142V transgenic mice which are hypertensive but do not have oxidative stress are not salt-sensitive even though they are on >98% C57B1/6J background (30, 34, 35) and D_1R function is impaired (30). Thus, the increase in ROS production mediates the salt-sensitive phenotype, at least, in the hGRK4γ486V transgenic mouse.

hGRK4γ486V transgenic mice have increased renal expression and activity of AT_1R on high salt diet (unpublished observations, Z.Wang). AT_1R has been shown to increase ROS production in the kidney via the NOX pathway (2, 3, 5, 6, 13, 31, 38). However, renal NOX1, NOX2, and NOX4 expressions are not increased in hGRK4γ486V mice; renal NOX2 is actually decreased in both hGRK4γ486V and Non-T mice, but to a greater extent in the latter than the former group. High salt diet slightly increases renal NOX activity in hGRK4γ486V transgenic mice and also tends to increase it in Non-T littermates such that NOX activity is not different between hGRK4γ486V transgenic mice and Non-T littermates. Therefore, NOX derived-ROS may not be the major cause of the increased oxidative stress in hGRK4γ486V transgenic mice. Moreover, renal AT_1R expression is also increased in hGRK4γ142V transgenic mice yet, these mice are not in a state of increased oxidative stress (27, 28). While an increase in salt intake increases the BP of C57Bl/6J mice (30, 35, 36) but not that of SJL mice (30), high salt intake also increases renal tubular AT_1R expression to the same degree in salt-resistant SJL and salt-sensitive C57Bl/6J mice (30) and salt intake increases renal ROS in the former but not in the latter group. Therefore, a salt-induced increase in renal AT_1R expression cannot explain the salt- induced increase in BP and oxidative stress in hGRK4γ486V transgenic mice.

Oxidative stress is caused not only by an increase in ROS production but also by a decrease in the scavenging of ROS. Thus, the renal protein levels of HO-1 and HO-2 were also quantified in the current studies because decreased renal protein abundance of HO-1 is associated with the hypertension of D5 dopamine receptor null mice (13) and decreased renal protein abundance of HO-2 is found in hypertensive D2 dopamine receptor null mice (12). However, the protein abundance of HO-1 is similarly decreased by high salt diet in hGRK4γ486V and Non-T mice. Renal HO-2 protein is actually higher in hGRK4γ486V than Non-T mice on normal salt diet and is decreased by high salt diet in hGRK4γ486V transgenic mice but to a level that is not different from Non-T littermates. Therefore, the decreased protein abundance and presumably activity of HO may not be the cause of the renal oxidative stress in hGRK4γ486V transgenic mice.

NO usually inhibits renal sodium transport and enhanced bioactivity of NO helps in the maintenance of normal BP (39, 40). The renal production of NO may be decreased by salt loading in hGRK4γ486V transgenic mice because NOS3 protein abundance is decreased in these mice on high salt diet. Urinary nitrite/nitrate concentration (Figure 5C) is similar in hGRK4γ486V and Non-T mice on normal salt diet and decreased by high salt diet to a greater extent in hGRK4γ486V than Non-T mice. Although a more sensitive measurement than urinary nitrite/nitrate for the quantification of renal NO production may be required, the decreased NOS3 expression in hGRK4γ486V could have caused the decrease in urine nitrite/nitrate concentration but not necessarily total NO production. Indeed, a decrease in the activity of one NOS isoform can be compensated by the other NOS isoforms resulting an unimpaired NO production (41–44). Actually, renal nitrotyrosine expression, the product of NO-superoxide interaction is increased (Figure 6) by high salt diet in both mouse strains, which may indicate a general increase in the renal production of ROS causing an increase in nitrotyrosine.

Decreased scavenging of ROS by SOD may be involved in the oxidative stress in hGRK4γ486V transgenic mice. High salt diet increases the renal protein abundance of MnSOD in Dahl salt-resistant but not Dahl salt-sensitive rats. Conversely, high salt diet also decreases renal CuZnSOD in Dahl salt- sensitive but not Dahl salt-resistant rats (23). Renal SOD activity and superoxide production are similar in hGRK4γ486V and Non-T mice on normal salt diet. However, high salt diet decreases renal SOD activity and increases superoxide production in hGRK4γ486V mice but not in Non-T mice. The current study also shows that the renal protein expressions of CuZnSOD and ECSOD are similar in hGRK4γ486V and Non-T mice on normal salt diet. However, high salt diet decreases CuZnSOD in hGRK4γ486V but not in Non-T mice and ECSOD is decreased to a greater extent in hGRK4γ486V than Non-T mice. By contrast renal MnSOD abundance is decreased in hGRK4γ486V mice to the same extent on normal and high salt diet, relative to Non-T mice. Of relevance is the finding that renal ECSOD is increased in salt-resistant hGRK4γWT mice, relative to the mildly salt-sensitive Non-T mice. We also find that tempol, a redox-cycling nitroxide and SOD mimetic (40), almost completely normalizes the SBP but completely normalizes urine 8-OHdG of hGRK4γ486V mice; 8-OHdG is another biomarker of oxidative stress (45). 8-OHdG is located in both the nucleus and cytoplasm, indicating that in the mouse kidney the oxidative DNA damage may occur not only in nuclei but also in mitochondria (46). Therefore, the decreased renal scavenging of superoxide by CuZnSOD and ECSOD, and possibly abetted by the decreased nitric oxide production by NOS3 may be the major contributors to the increased oxidative stress in the hGRK4γ486V mice. Activation of the RAS generally causes an increase in ROS production (2, 3, 5, 6). However, we did not find any difference in serum renin and urine aldosterone (data not shown) between the hGRK4γ486V mice and Non-T littermates.

GRK4 regulates several G protein-coupled receptors (GPCRs), including the D_1R and D_3R (1, 5, 20, 21, 24, 25, 27–30). The D_2R and D_5R are important in the maintenance of normal redox balance by inhibiting phospholipase D2 and NOX expression and activity and by stimulating paraoxonase 2, HO- 1, and HO-2 activities (11–13). The D_3R can also increase renal SOD activity but the specific SOD isoform involved was not determined (47). We have suggested that the salt sensitivity of C57Bl/6J mice is caused by impaired function of renal

D_1-like receptors (comprised of D_1R and D_5R), due to increased GRK4 expression, and decreased renal dopamine production but not by increased renal oxidative stress (30). D_1-like receptor function can be impaired by hGRK4γ142V or hGRK4γ486V (1, 5, 20, 21, 25, 27–29). However, as stated above, hGRK4γ142V transgenic mice are hypertensive on normal salt diet while hGRK4γ486V transgenic mice are hypertensive only on high salt diet. We have reported that hGRK4γ142V impairs D_1R function to a greater than hGRK4γ486V (29) and that impaired D_1R function leads to an increase in BP (1, 5, 20, 21, 25, 27–29) without renal oxidative stress (27). Therefore, it is possible that the impairment of D_1R function, magnified by the increase in renal ROS production, causes the salt-sensitive hypertension of hGRK4γ486V transgenic mice.

In summary, hGRK4γ468V transgene causes salt sensitivity in mice that is associated with increased renal oxidative stress. The increased oxidative stress is caused mainly by decreased SOD activity that may be secondary to the decreased protein abundance of CuZuSOD and ECSOD. Based on the current findings, reduction in salt intake and antioxidant treatment may benefit the hypertensive patients who are carriers of the hGRK4γ486V variant (18, 19). hGRK4γ486V is associated with increased cardiovascular risk (48) but may increase the response to diuretic therapy (49). Only a few gene variants thought to be causal of hypertension in humans have been shown to produce hypertension in mice, e.g., AGT that encodes angiotensinogen (50), AGTR1 that encodes the AT_1R (51), CYP11B2 that encodes aldosterone synthase (52), and UMOD (53) that encodes uromodulin. However, GRK4 is the only human gene in which the hGRK4γWT transgene is associated with normal BP and salt resistance, the hGRK4γ142V transgene causes salt-resistant hypertension while the hGRK4γ486V causes salt-sensitive hypertension (normal BP on normal sodium diet and high BP on high salt diet) in mice. Moreover, hGRK4 gene variants can predict the response to antihypertensive drugs in humans (25, 48, 49, 54–57), an example of pharmacogenomics for hypertension.

INNOVATION

In humans, hGRK4γ486V is associated with salt-sensitive hypertension. We used hGRK4γ486V transgenic mice to determine its role in salt-sensitive hypertension. hGRK4γ486V transgenic mice are salt-sensitive and in a state of increased renal oxidative stress, that is related to decreased renal protein abundance of CuZnSOD, ECSOD, and NOS3. By contrast, hGRK4γ142V mice are hypertensive but salt-resistant, while hGRK4γWT mice are normotensive and salt-resistant; the two latter mouse strains are in normal redox balance. Tempol normalizes urine 8-OHdG and partially prevents the salt-sensitive hypertension of hGRK4γ486V mice. This is the first demonstration in mice that a wild-type gene, human GRK4γ, causes salt resistance, while its variants cause salt-resistant (GRK4γA142>V) and salt-sensitive (GRK4γA486>V) hypertension.

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ABBREVIATIONS

8-OHdG	8-hydroxydeoxyguanosine
142V	hGRK4γ142V
486V	hGRK4γ486V
AT_1R	angiotensin II type I receptor
BP	blood pressure
CuZnSOD	copper-zinc superoxide dismutase
ECSOD	extracellular superoxide dismutase
hGRK4	human G protein-coupled receptor kinase 4
HO	heme oxygenase
HS	high salt
MnSOD	manganese superoxide dismutase
NO	nitric oxide
Non-T	nontransgenic
NOS	nitric oxide synthase
NOX	NADPH oxidase
NS	normal salt
RAAS	renin-angiotensin-aldosterone system
ROS	reactive oxygen species
SBP	systolic blood pressure
SOD	superoxide dismutase

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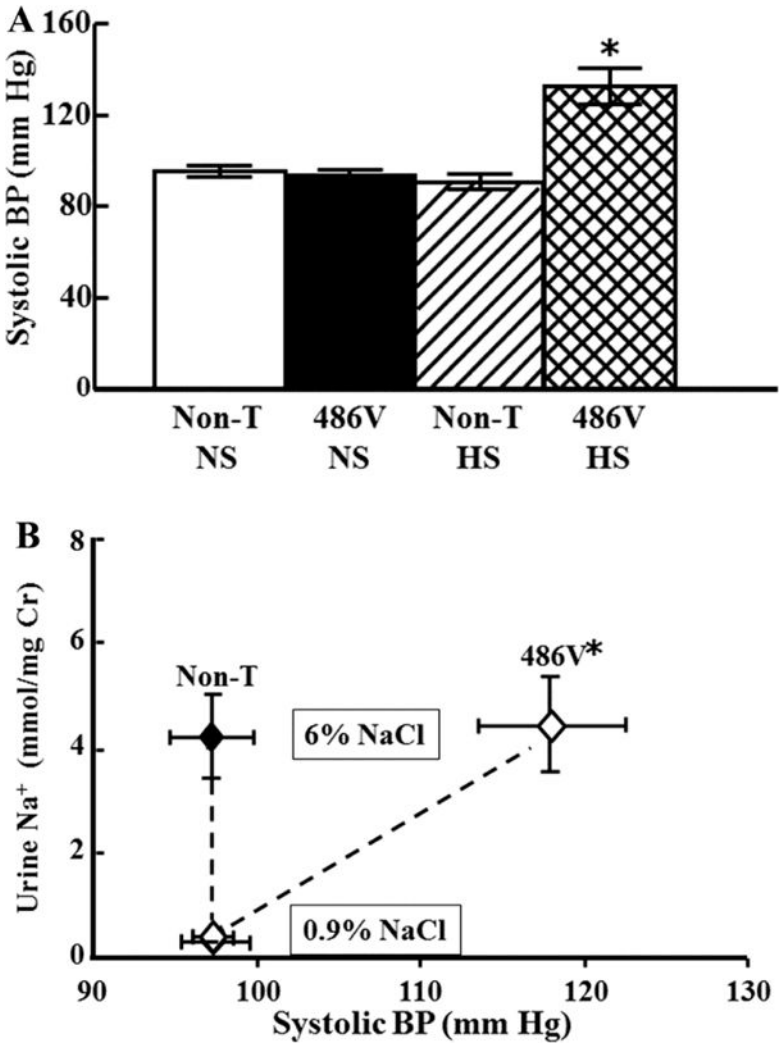

Figure 1.

Blood pressure (BP) and pressure-natriuresis plot in response to high salt diet. **1A.** Effect of high salt diet on BP. Systolic BP was measured under pentobarbital anesthesia. SBP is similar in hGRK4γ486V (486V) and Non-T (Non-T) littermates on normal salt (NS, 0.9% NaCl) diet. SBP is increased by high salt (HS, 6% NaCl) diet in 486V transgenic mice but not Non-T littermates. n=4–5/group, *P<0.05 vs others, one-way factorial ANOVA, Holm-Sidak test. **1B.** Additional mice were placed in metabolic cages for 24 hr urine collection. SBP was measured under anesthesia after the urine samples were collected. Urinary sodium excretion (Urine Na^+) is corrected by urine creatinine (Cr). On NS diet, SBPs are similar in 486V (89.83±2.7, mm Hg) mice and their Non-T (94.7±2.5) littermates. Systolic BP in 486V, not in Non-T mice, is increased by HS diet. Urine Na^+ is similar on NS diet and increased similarly in the two mouse strains by HS diet. However, the pressure-natriuresis plot of 486V mice is shifted to the right of the Non-T littermates, indicating an impairment in the ability to excrete the salt load. n=5–9/group, *P<0.05 vs others, one-way factorial ANOVA, Holm-Sidak test.

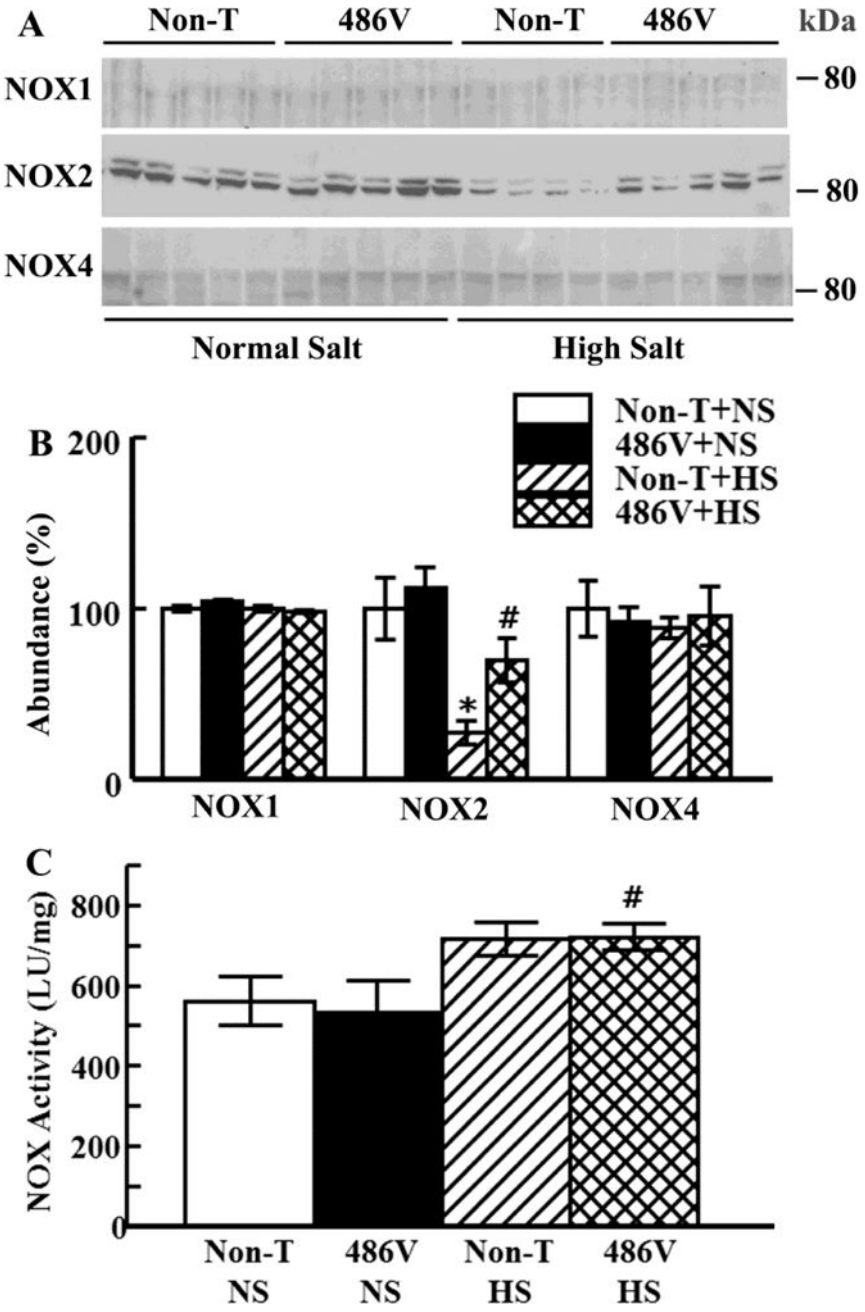

Figure 2.

Renal NOX protein abundance and activity. **2A**. Immunoblots of NOX1, NOX 2, and NOX4 in whole kidney homogenates (n=4–5/group). **2B**. Densitometric analysis of the immunoblots. The renal protein abundances (band densities as *%* of non-transgenic [Non-T] mice on normal salt [NS, 0.8% NaCl] diet) of NOX1, 2, and 4 are similar in hGRK4γ486V and Non-T mice on NS diet; NOX2 is decreased by high salt (HS, 6% NaCl) diet in both strains but to lesser extent in 486V mice than Non-T littermates (70±13 vs 28±7%). N=5–6/group, *P<0.05 vs others, #P<0.05 vs NS diet, one-way factorial ANOVA, Holm-Sidak test. **2C.** Total NOX activity in whole kidney homogenates is similar in 486V and Non-T mice on

NS (533±82 vs 561±61, LU/mg protein) and HS diets (722±3 vs 718±42). HS diet slightly increases total renal NOX activity in both mouse strains, reaching significance in 486V mice. N=5–6/group, $^{\#}P<0.05$ vs 486V+NS diet, one-way factorial ANOVA, Holm-Sidak test.

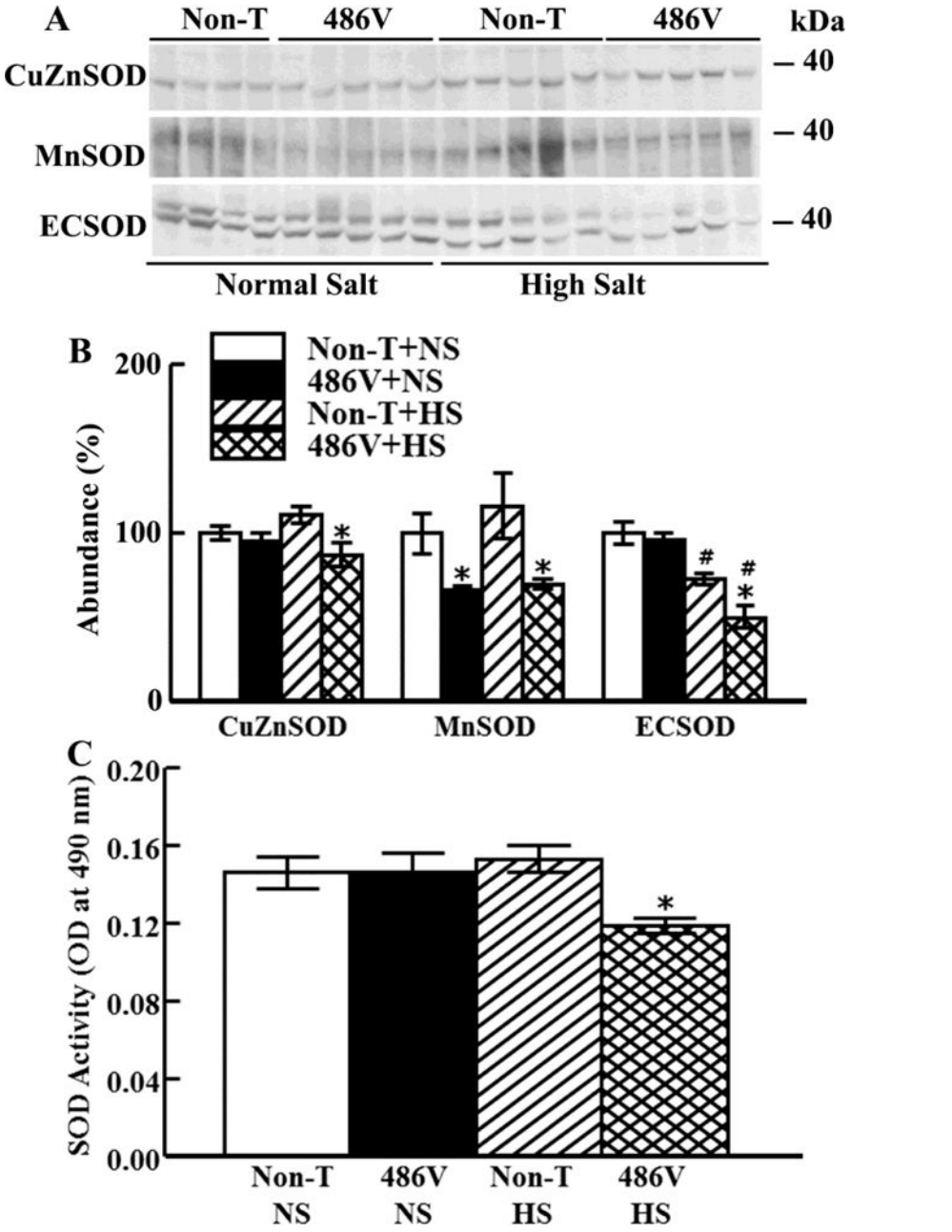

Figure 3.

Renal superoxide dismutase (SOD) protein abundance and activity. **3A.** Immunoblots of CuZnSOD, MnSOD, and ECSOD in whole kidney homogenates (n=4–5/group). **3B.** Densitometric analysis of the immunoblots. On NS (0.8% NaCl) diet, the renal protein abundances of CuZnSOD and ECSOD are similar in the hGRKγ486V (486V) transgenic mice and Non-transgenic (Non-T) littermates while MnSOD (66±3%) is lower in 486V than Non-T mice. On HS (6% NaCl) diet, CuZnSOD (87±7%) is decreased in 486V but not Non-T mice; HS diet decreases ECSOD (55±7%) to a greater extent in 486V mice than Non-T mice. On HS diet, MnSOD (70±3%) remains lower in 486V than Non-T mice but not different from 486V mice on NS diet. *P<0.05 vs Non-T mice, #P<0.05 vs NS diet, one-way

factorial ANOVA, Holm-Sidak test. **3C.** Total SOD activity in whole kidney homogenates is similar in 486V (0.146±0.01) and Non-T (0.146±0.008) mice on NS diet. Total SOD activity is decreased by HS diet in 486V mice (0.119±0.004) but not Non-T littermates (0.153±0.007). *P<0.05 vs other groups, one-way factorial ANOVA, Holm-Sidak test.

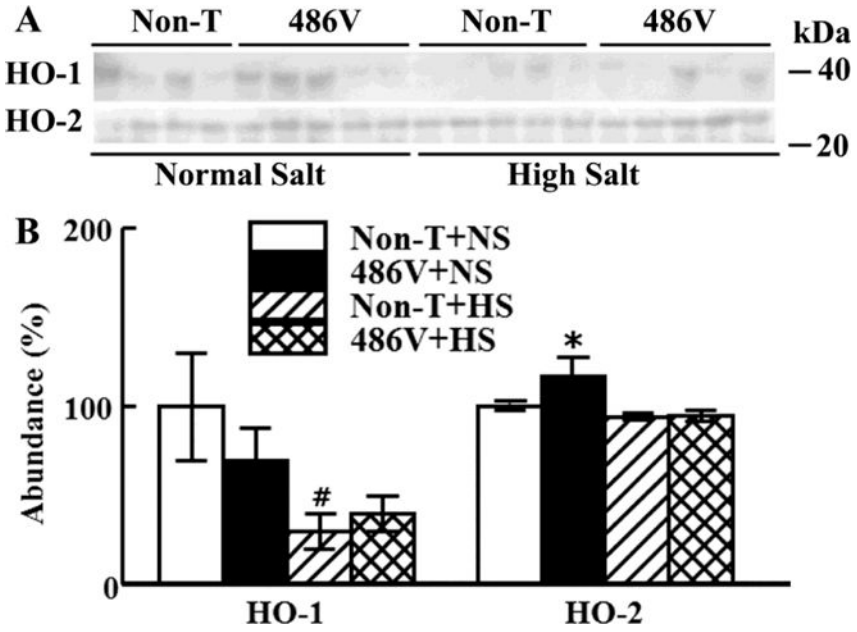

Figure 4.

Renal heme oxygenase (HO) protein abundance. **4A**. Immunoblots of HO-1 and HO-2 in whole kidney homogenates (n=4–5/group). **4B**. Densitometric analysis of the immunoblots. On NS (0.8% NaCl) diet, the renal protein abundance of HO-1 is similar in hGRK4γ486V (486V) transgenic mice and their Non-transgenic (Non-T) littermates. HS (6% NaCl) diet decreases HO-1 protein abundance in both 486V and Non-T mice, reaching significance in Non-T mice. The protein abundance of HO-2, which is slightly greater in 486V (117±7%) than Non-T mice on NS diet, is decreased in 486V but not Non-T mice by HS diet. $^{\#}P<0.05$ vs Non-T NS, *P<0.05 vs others, one-way factorial ANOVA, Holm-Sidak test.

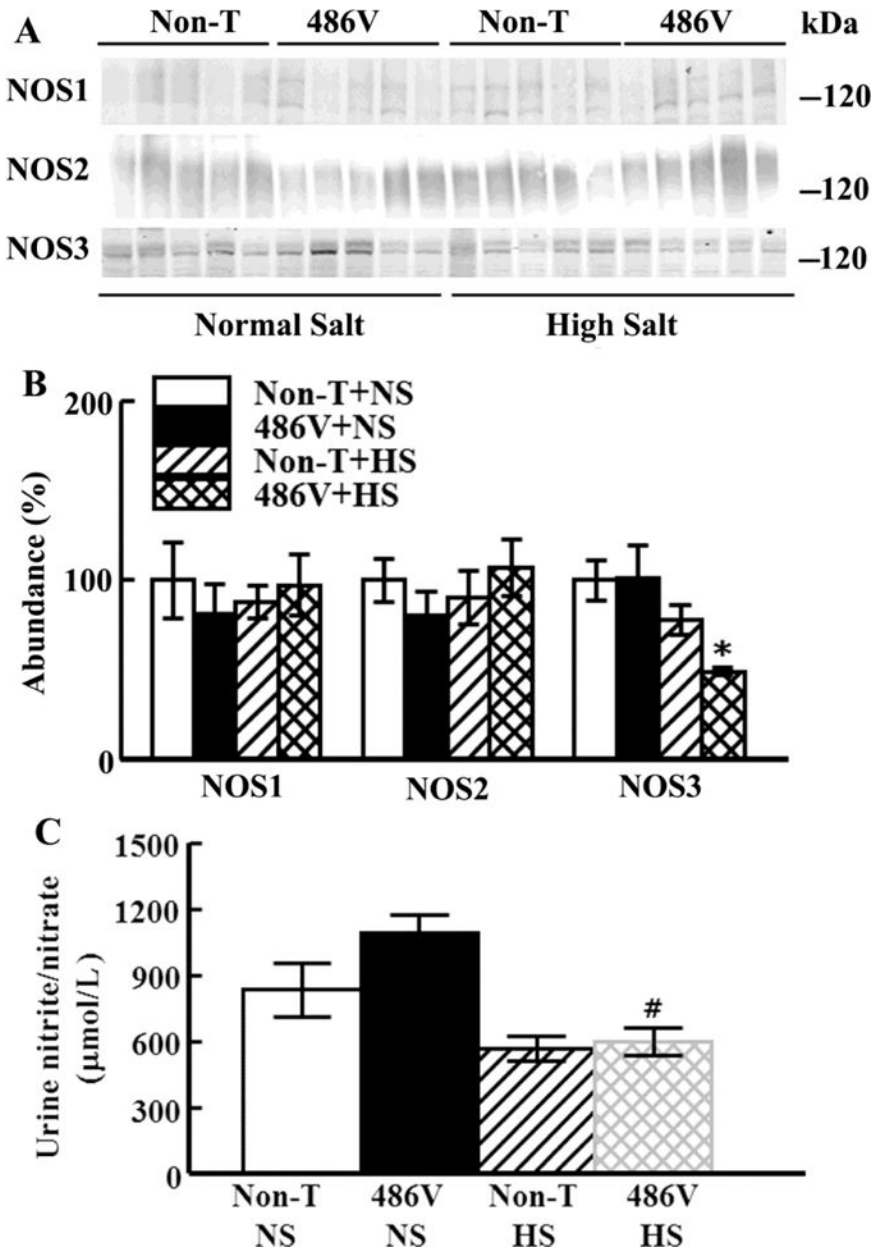

Figure 5.

Renal nitric oxide synthase (NOS) protein abundance and urinary nitrite/nitrate concentration. **5A**. Immunoblots of NOS1, NOS2, and NOS3 in whole kidney homogenates (n=5/group). **5B**. Densitometric analysis of the immunoblots. On NS (0.8% NaCl) diet, the renal protein abundances of NOS1 and NOS2 in GRK4γ486V (486V) transgenic mice and Non-transgenic (Non-T) littermates are similar and not altered by HS (6% NaCl) diet. NOS3 protein abundance is similar in 486V and Non-T mice on NS diet but is decreased by HS diet in 486V (49±2%) but not Non-T mice. *P<0.05 vs other groups, one-way factorial ANOVA, Holm-Sidak test. **5C.** Urinary nitrite/nitrate concentration is similar in 486V (163±15, μmol/mg of Cr, n=7) and Non-T (161±11, n=6) littermates on NS diet and

decreased by HS diet, reaching significance in 486V mice. #P<0.05 vs NS diet, one-way factorial ANOVA, Holm-Sidak test.

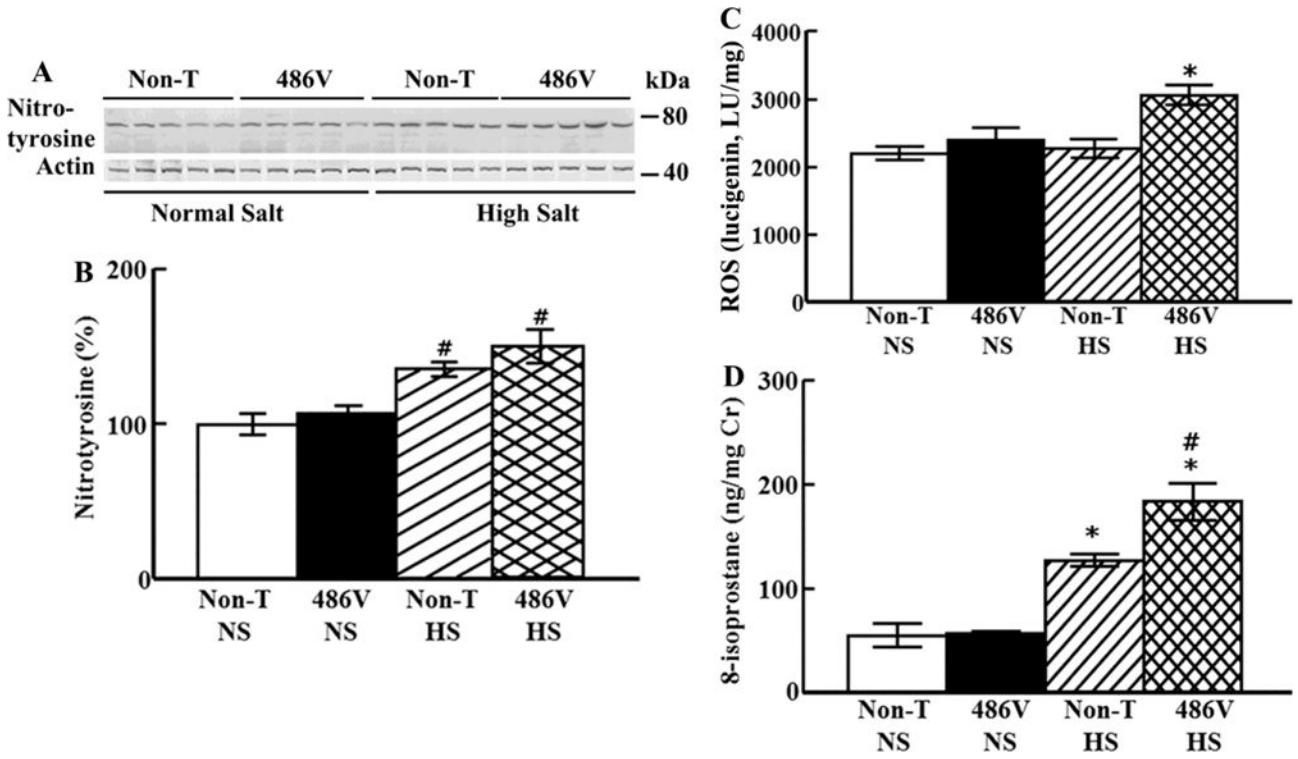

Figure 6.

Renal nitrotyrosine abundance, ROS production, and urinary 8-isoprostane excretion. **6A**. Immunoblots of nitrotyrosine and actin in whole kidney homogenates (n=5/group). **6B**. Densitometric analysis of the immunoblots. On NS (0.8% NaCl) diet, the renal protein abundance of nitrotyrosine is similar in hGRK4γ486V transgenic mice and is increased similarly in 486V (150±11%) and Non-T (138±5%) mice by HS (6% NaCl) diet. $^{\#}P<0.05$ vs NS diet, one-way factorial ANOVA, Holm-Sidak test. **6C**. Reactive oxygen species (ROS) production in whole kidney homogenates. Renal ROS production is similar 486V and Non-T mice on NS diet (2401±179 vs 2201±102, LU/mg protein). HS diet increases ROS production only in 486V mice (3061±145 vs 2276±139). n=3–4/group, *P<0.05 vs other groups, one-way factorial ANOVA, Holm-Sidak test. **6D**. Urinary 8-isoprostane excretion, corrected by creatinine (Cr), is similar in 486V and Non-T mice on NS diet. HS diet increases urinary 8-isoprostane excretion to a greater extent in 486V mice than Non-T littermates (197.3±17.7 vs 127.6±5.9, respectively). n=3–4/group, *P<0.05 vs NS diet, $^{\#}P<0.05$ vs Non-T+HS diet, one-way factorial ANOVA, Holm-Sidak test.

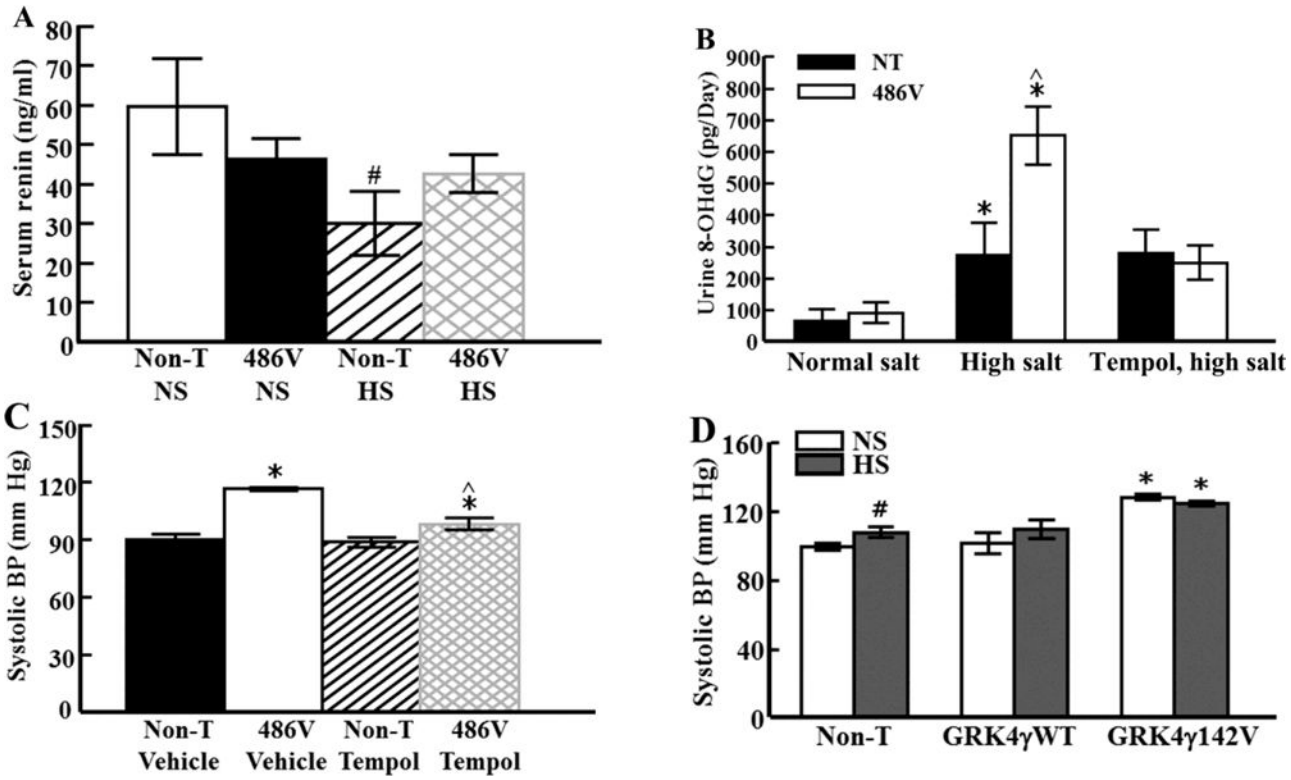

Figure 7.

Serum renin, urine 8-hydroxydeoxyguanosine (8-OHdG), tempol treatment, and salt sensitivity. **7A**. Serum renin concentration is similar in 486V and Non-T mice on NS diet (46.42±5.24 vs 59.74±12.11). HS diet decreases serum renin concentration in Non-T littermates (30.09±8.17) but not 486V mice (42.62±0.09). n=4/group, $^{\#}P<0.05$ vs Non-T +NS diet, one-way factorial ANOVA, Holm-Sidak test. **7B**. Urinary 8-OHdG excretion is similar in 486V and Non-T mice on NS diet (91.4±33.2 vs 66.2±37.4). HS diet increases urinary 8-OHdG excretion to a greater extent in 486V (651.7±91.7) than Non-T mice (273.9±103.7). Tempol, added into drinking water (1 mmol/L, 2 weeks), normalizes urinary 8-OHdG excretion in 486V mice (250.6±54.4 vs 281.8±73.4). n=4/group, *P<0.05 vs NS diet, ^P<0.05 vs tempol+HS diet, one-way factorial ANOVA, Holm-Sidak test. **7C**. Systolic BP, measured in the aorta, via the femoral artery, under anesthesia, is higher in hGRK4γ486V than Non-T mice after 1 week of HS diet. Tempol, added into drinking water (1 mmol/L, 2 weeks), decreases BP in 486V but not Non-T mice fed HS for 3 weeks. However, the systolic BP is still slightly higher in 486V than Non-T mice. **7D**. Systolic BP, measured under anesthesia, is similar in hGRK4γWT and their Non-transgenic (Non-T) littermates but lower than that measured in hGRK4γ142V transgenic mice on NS (0.8% NaCl) diet; all mice are on >98% C57Bl/6J background. HS (6% NaCl) diet slightly increases SBP in Non-T mice but not in hGRK4γWT mice and does not increase the already elevated SBP of hGRK4γ142V transgenic mice. 4–5 months old, mixed sex, n=4–7/group. *P<0.05 vs others, $^{\#}P<0.05$ vs NS, Non-T, one-way factorial ANOVA, Holm-Sidak test.

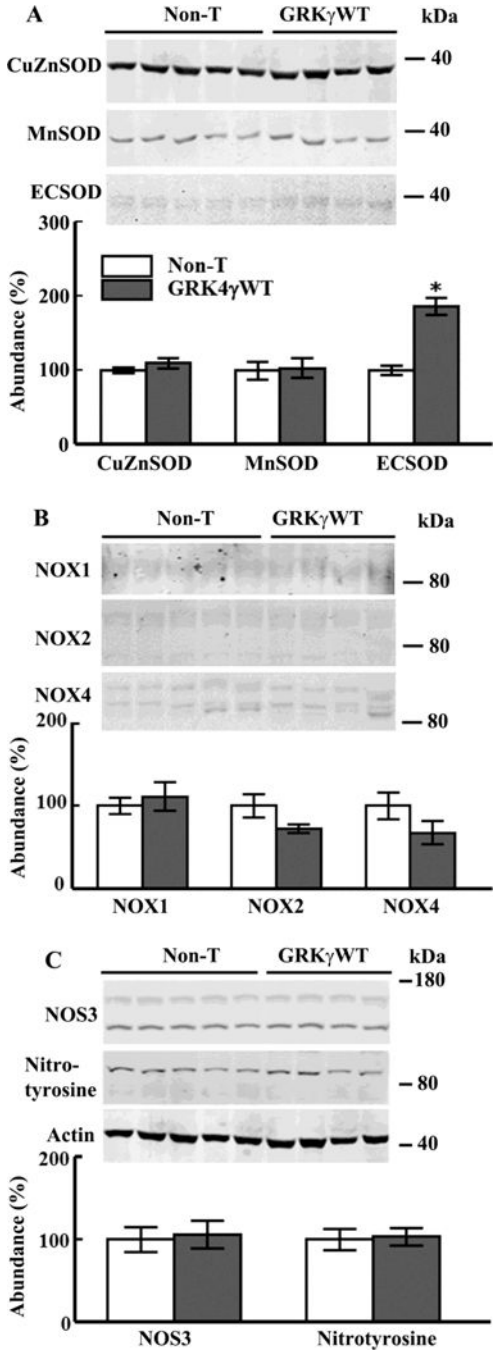

Figure 8.

Renal ROS-related proteins in hGRK4γWT transgenic mice and Non-transgenic (Non-T) littermates on high salt (HS, 6% NaCl) diet. **8A.** Immunoblots of SOD isoforms in whole kidney homogenates (n=4–5/group). Densitometric analysis of the immunoblots. The protein abundances of CuZnSOD and MnSOD are similar in hGRK4γWT and Non-T littermates but ECSOD is increased in hGRK4γWT transgenic mice (186±11%), relative to Non-T littermates (100±6%). n=4–5/group, Student's t-test. **8B.** Immunoblots of NOX1, NOX2, and NOX4 in whole kidney homogenates (n=4–5/group)._ Densitometric analysis of the

immunoblots. The protein abundances of all three NOX isoforms are similar in hGRK4γWT and Non-T littermates. n=4–5/group, Student's t-test. **8C.** Immunoblots of NOS3, nitrotyrosine, and actin in whole kidney homogenates (n=4–5/group). Densitometric analysis of the immunoblots. The protein abundances of NOS3 and nitrotyrosine are similar in hGRK4γWT and Non-T littermates. n=4–5/group, Student's t-test.

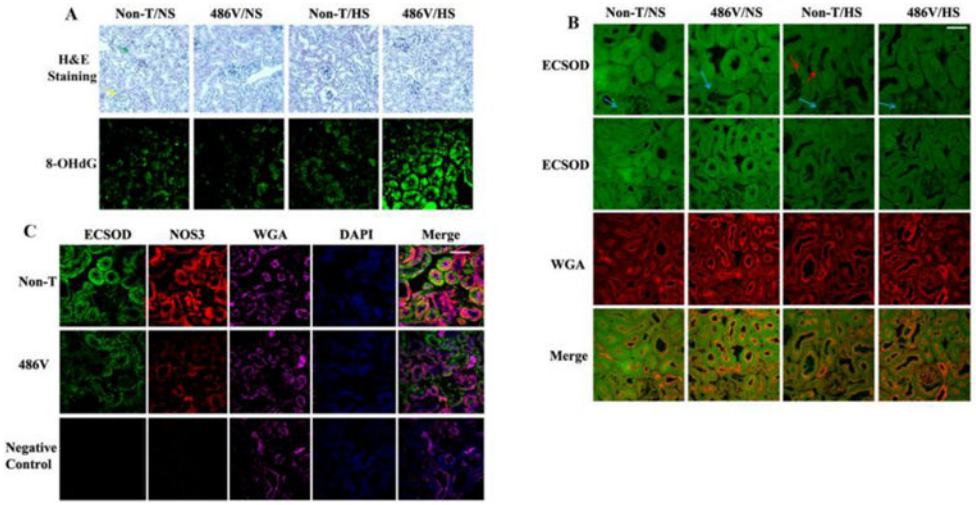

Figure 9.

Kidney structure and immunostaining of 8-OHdG, ECSOD, and NOS3 in hGRK4γ486 (486V) transgenic mice and Non-transgenic (Non-T) littermates. **9A.** H&E staining of mouse kidney (**upper panel**) and 8-OHdG immunostaining in mouse renal cortex (**lower panel**). hGRK4γ486 (486V) mice and Non-T (non-T) littermates were fed normal NS (0.8% NaCl) or HS (6% NaCl) diet. The H&E staining is similar in the two groups. 8-OHdG immunostaining in mouse kidney cortex (red) appears stronger in hGRK4γ486/HS than the other groups. Scale=40 μM. Green arrows: proximal tubule; Yellow arrows: glomerulus. **9B.** ECSOD immunostaining in mouse kidney cortex. ECSOD immunostaining (1:100, rabbit, green) is observed in renal tubules and glomeruli. The magnification is 400× except for the images on the top row (600×). More ECSOD is expressed in renal tubules than glomeruli (blue arrows). Strong staining is observed in apical membranes (red arrows). The signal strength of ECSOD is decreased by HS in both mouse strains. Wheat germ agglutinin (WGA, red) stains plasma membrane. Scale=20 μM. **9C.** ECSOD and NOS3 co-immunostaining in mouse kidney cortex of 486V mice and Non-T littermates fed HS. More ECSOD antibody (1:50, rabbit, green) is used to take into account the reduced staining observed with HS diet. More ECSOD is expressed in renal tubules than glomeruli while NOS3 (1:100, mouse, red) is similarly expressed in renal tubules and glomeruli. Colocalization of ECSOD and NOS3 (yellow and orange in the merge image) is observed mainly in renal tubules which is more evident in Non-T than 486V mice. The negative controls were not stained with the primary antibodies. WGA (magenta) was used to stain the plasma membrane; DAPI (blue) was used to stain the nucleus. Scale=20 μm.

Table 1

Physiological data in mice fed normal and high NaCl diets with free access to water and food for 3 weeks

Group	NT, NS	486, NS	NT, HS	486, HS
Body weight, before (g)	27.8±1.8	27.9±1.7	26.4±1.2	24.1±0.7
Body weight, after (g)	28.4±2.0	27.4±1.7	26.8±1.3	23.5±0.8
Water intake (ml/day)	4.66±1.17	4.28±0.31	9.00±1.10 *	10.50±3.20 *
Food intake (g/day)	3.76±0.34	4.58±0.47	10.90±1.40 *	11.80±1.50 *
Urine (ml/day)	1.78±0.43	1.22±0.23	3.00±0.50 *	3.20±0.40 *
Urine sodium (mmol/day)	0.32±0.08	0.25±0.02	1.38±0.24 *	1.60±0.14 *
Urine potassium (mmol/day)	0.62±0.16	0.46±0.03	0.27±0.04 *	0.30±0.03 *
Urine chloride (mmol/day)	0.44±0.11	0.34±0.03	1.41±0.24 *	1.60±0.16 *
Urine creatinine (mg/dl)	0.57±0.08	0.55±0.03	0.10±0.02 *	0.13±0.03 *
Urine sodium/creatinine (mmol/Cr mg)	0.32±0.02	0.40±0.03	1.81±0.35 *	1.75±0.04 *
Serum sodiun (mmol/L)	139.9±4.2	140.1±3.9	147.8±1.2	147.4±0.8
Serum potassium (mmol/L)	4.20±0.32	3.80±0.09	4.60±0.40	4.70±0.30 *
Serum chloride (mmol/L)	120.7±3.4	123.7±4.9	130.8±1.8	127.8±1.5

Data are shown as mean ± standard error. n=5/group.

* P<0.05 high (HS) vs normal NaCl (NS) diet, One-way factorial ANOVA and Holm-Sidak post-hoc test. NT: non-transgenic mice, 486: hGRK4γ486V transgenic mice.