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Regulation of blood pressure, oxidative stress and AT1R by high salt diet in mutant human dopamine D5 receptor transgenic mice

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Abstract

Humans have dopamine D5 receptors (hD5R) with single-nucleotide polymorphisms and a diminished function. We generated *hD5^{F173L}* cDNA that has a decreased response to D5R agonist-mediated increase in cAMP production and increased production of reactive oxygen species, relative to wild-type hD5R (*hD5^{WT}*) cDNA expressed in Chinese hamster ovary cells. To investigate the role of *hD5^{F173L}* in the pathogenesis of salt-sensitive hypertension, we generated transgenic mice overexpressing *hD5^{F173L}* or *hD5^{WT}* and fed them normal (0.8% NaCl) or high (4% NaCl) salt diet. On normal salt diet, the blood pressure, and renal NADPH oxidase activity and angiotensin type 1 receptor (AT1R) expression were higher in *hD5^{F173L}* than *hD5^{WT}* transgenic mice. After 2 weeks on high salt diet, the blood pressure and renal NADPH oxidase activity, but not AT1R expression, were increased in *hD5^{F173L}* but not in *hD5^{WT}* transgenic mice. Candesartan, an AT1R antagonist, decreased the blood pressure and NADPH oxidase activity in *hD5^{F173L}* but not in *hD5^{WT}* transgenic mice. We suggest that the ability of the hD5R to negatively regulate the renal NADPH oxidase activity and AT1R function may have important implications in the pathogenesis of salt-sensitive blood pressure. However, the mechanisms involved in regulating the balance of renal D5R and AT1R function in the oxidative stress-mediated salt-sensitive blood pressure remain to be determined.

Keywords

D5R; AT1R; NADPH oxidase; salt-sensitive hypertension

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INTRODUCTION

Hypertension is a multifactorial disorder resulting from the interactions between environmental and genetic factors.^{1,2} Epidemiological and interventional studies have demonstrated a clear relationship between salt intake and hypertension.³ Reactive oxygen species (ROS) are also known to be involved in the development of salt-sensitive hypertension and renal disease.^{4,5} A major source of ROS in the kidney is nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NAD(P)H) oxidase, which is an enzyme complex consisting of six subunits: membrane subunits p22^{phox} and gp91^{phox}, cytosolic components p40^{phox}, p47^{phox} and p67^{phox} and a low-molecular weight G protein rac1 or rac2. Several homologues of gp91^{phox} in mammalian non-phagocytic cells have been reported, including Nox 1, Nox 2, Nox 3, Nox 4 and Nox 5; Nox 5 is present in humans but not rodents. The rodent kidney mainly expresses Nox 1, Nox2 and Nox 4, that act as O₂⁻-producing NAD(P)H oxidases.^{6,7}

Abnormalities in dopamine production and receptor function have been described in human essential hypertension and rodent models of genetic hypertension.^{2,8-11} The effects of dopamine are exerted by two families of cell surface receptors that belong to the superfamily of G protein-coupled receptors; D₁-like receptors (D1R and D5R) stimulate adenylyl cyclases, whereas D₂-like receptors (D2R, D3R and D4R) inhibit adenylyl cyclases.^{2,8-11} Compared with D1R, the D5R has a higher affinity for dopamine, and exhibits constitutive activity.^{10,11} The locus of the human D5R (hD5R), 4p15.1-16.1, and its pseudogenes, 2p11.1-p11.2, 1q21.1, have been linked to human essential hypertension.¹²⁻¹⁴ Moreover, humans have D5R gene single-nucleotide polymorphisms with a diminished function.¹⁵

We have reported that the disruption of the D5R gene (*Drd5*) in mice increases blood pressure. The elevated blood pressure in *Drd5*^{-/-} mice was suggested to be caused, in part, by an increased ROS production via an increased renal NADPH oxidase and phospholipase D activities.^{10,11} Dopamine, via its receptors, including the D5R, decreases renal tubular sodium transport. Decreasing the renal dopamine production or dopamine receptor expression or function results in sodium retention, contributing to the development of hypertension.^{2,8-11,16-21} However, the regulation of sodium transport by dopamine and its receptors also involves interaction with other G protein-coupled receptors.¹⁶ Indeed, the negative interaction between D5R and angiotensin type 1 receptor (AT1R) is involved in the regulation of renal sodium transport and pathogenesis of hypertension.^{16,22,23} We hypothesize that hD5R negatively regulates renal NADPH oxidase and AT1R function that may be important in the normal regulation of blood pressure. Moreover, impaired hD5R function may result in salt-sensitive hypertension.

To investigate the role and mechanism of D5R-mediated regulation of blood pressure and salt sensitivity, we generated mutant D5R with a single-nucleotide polymorphism, *hD5*^{F173L}. We transfected the *hD5*^{F173L}, and hD5 wild-type (*hD5*^{WT}) cDNAs into Chinese hamster ovary (CHO) cells and tested their ability to regulate cyclic adenosine monophosphate (cAMP) and ROS production, in response to a D1R/D5R agonist, fenoldopam, which in the absence of D1R is a selective D5R agonist and vice versa. After establishing that *hD5*^{F173L}, relative to *hD5*^{WT}, has a decreased ability to increase cAMP production and negatively

regulates ROS production, hD5^{F173L} and hD5^{WT} transgenic mice were generated that were studied for the effect of high salt intake on renal oxidative stress, AT1R expression and blood pressure.

METHODS

Materials

Cytochrome C, diphenyleneiodonium, fenoldopam, SCH23390, superoxide dismutase and candesartan were purchased from Sigma-Aldrich, St. Louis, MO, USA. NADPH was from ICN Biomedicals, Aliso Viejo, CA, USA. Lucigenin was from Molecular Probes (Grand Island, NY, USA), Invitrogen (Carlsbad, CA, USA), Eugene (Shanghai, China). Polyclonal AT1 antibody (N-10) and monoclonal anti-Nox 2 and Nox 4 antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

hD5^{WT} and hD5^{F173L} cell transfections

The full-length hD5^{WT} and hD5^{F173L} cDNAs, subcloned into a pcDNA6/V₅-His vector between the EcoRI and XbaI sites, respectively, were transfected into CHO cells using LT1 transfection reagents.¹⁰ The successful transfection of hD₅ cDNA was verified by immunoblotting for His/V₅ expression. Empty vector-transfected CHO cells served as negative controls.

Cyclic AMP accumulation assay

The CHO cells, heterologously expressing hD5^{WT}, hD5^{F173L} or empty vector were grown to 70% confluence in six-well plates and pre-treated with 1 μM phorbol 12-myristate 13-acetate for 5 min in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 μM) before the D1R/D5R agonist fenoldopam (10 nM, 100 nM, 1 μM) treatment for 0 and 30 mins; in the absence of D1R, fenoldopam is a specific D5R agonist. Cell lysates were prepared to determine protein concentration by using the BCA protein assay kit (Thermo-Scientific, Rockford, IL, USA) and cAMP concentration by using the Direct Cyclic AMP Enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA), following the manufacturer's procedures. All assays were performed in duplicates.

Detection of ROS

The oxidation of 2',7'-dichlorodihydrofluorescein diacetate was used to measure ROS in the CHO cells heterologously expressing hD5^{WT}, hD5^{F173L} or empty vector. To test the effect of cellular NADPH oxidase inhibition on ROS production, cells were pre-treated with an inhibitor of NADPH oxidase (diphenyleneiodonium (DPI), 10 mM per 30 min) (Sigma), and then treated with H₂O₂ (50 μM per 2 h, ThermoScientific) or vehicle. The cells were incubated with fresh 2',7'-dichlorodihydrofluorescein diacetate (10 mM per 30 mins) at 37 °C. 2',7'-Dichlorodihydrofluorescein diacetate fluorescence was measured using a microplate reader in 96-well plates at an excitation wave length of 485nm and an emission wave length of 530nm. ROS production was expressed in arbitrary units corrected for the protein concentration (AU mg⁻¹ protein). All assays were performed in duplicate.

Transgenic mice and blood pressure measurement

The full-length *hD5^{WT}* and *hD5^{F173L}* cDNAs were each subcloned into an expression plasmid under the CMV promoter. The transgenic mice were generated by the microinjection method in oocytes. The genotype of the transgenic mice was verified by polymerase chain reaction using the primers, 5'-GGACCGCTACTGGGCCATCT-3' and 5'-GGGTCTTGAGAACCTTGGTC-3', and the sequence of the amplified 488 bp fragment of *hD5R* was analyzed.

The mice (C57Bl/6) were fed a normal NaCl diet (0.8%) for 14 days that was subsequently changed to a high NaCl diet (4%) for 15 days, and then injected intraperitoneally with candesartan (AT1R antagonist, 1 mg kg⁻¹ body weight per day) or vehicle (saline, control) daily for 10 days. Thereafter, blood pressures were measured from the aorta via the femoral artery under pentobarbital anesthesia.^{10,11} The kidneys were harvested, immunoblotted for AT1R, Nox2 and Nox4 and assayed for NADPH oxidase activity.

NADPH oxidase activity assay

NADPH oxidase activity (light units per g protein per minute) of membrane proteins from mouse kidney tissues was measured by NADPH-induced chemiluminescence with 5 μmol l⁻¹ lucigenin and 100 μmol l⁻¹ NADPH.¹¹ The specificity of the NADPH-dependent O₂⁻ production was verified by treatment with the flavoprotein inhibitor diphenyleneiodonium.

Immunoblotting

Mouse kidney homogenates were prepared for immunoblotting as previously reported.¹¹ The samples were immunoblotted with well characterized anti-Nox2, anti-Nox4 and anti-AT1R antibodies. Uniformity of protein loading and membrane transfer was determined by immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GADPH).

Statistical analysis

Data are expressed as mean±s.e.m. Comparison of more than two groups was made by one-way or two-way factorial analysis of variance, Holm–Sidak test and a comparison between two groups utilized the *t*-test. A value of *P*<0.05 was considered statistically significant.

RESULTS

cAMP and ROS production in CHO cells

Basal cAMP levels in plasmid-transfected CHO cells (0.54±0.02 pmol mg⁻¹ protein per min) were similar to CHO cells expressing *hD5^{F173L}* (0.52±0.03), but less than that noted in CHO cells expressing *hD5^{WT}* (0.84±0.03) (*P*<0.05, one-way analysis of variance, Holm–Sidak test). The higher basal cAMP levels in the *hD5^{WT}* indicate that the D5R receptor is constitutively active, in agreement with previous reports,¹⁰ Fenoldopam (10⁻⁸–10⁻⁶ M, 30 min) significantly increased cAMP production in *hD5^{WT}* and but not in *D5^{F173L}* or vector-transfected cells (Figure 1).

We next determined the ability of *hD5R* to regulate ROS production. In the basal state, ROS production was similar in plasmid-transfected CHO cells (39300±200 arbitrary units/mg

protein), CHO cells expressing hD5^{F173L} (38500±120) and CHO cells expressing hD5^{WT} (39300±200). The ability of 50 mM H₂O₂ to increase ROS production was greater in CHO cells transfected with plasmid (44900±200) or hD5^{F173L} (44500±300) than CHO cells transfected with hD5^{WT} (41200±80) (Figure 2).

Blood pressures in hD5R transgenic mice

On normal salt diet (0.8% NaCl×14 days), the systolic, diastolic and mean arterial blood pressures, measured under pentobarbital anesthesia, were higher in 3-month-old hD5^{F173L} than hD5^{WT} transgenic mice (Figure 3a). A high salt diet (4% NaCl×15 days), increased further the blood pressures in hD5^{F173L} but had no effect in hD5^{WT} mice. The high blood pressures of hD5^{F173L} transgenic mice on high salt diet were normalized after 10 days of candesartan, an AT1R antagonist, which had no effect in hD5^{WT} transgenic mice (Figure 3b).

Expression of NADPH oxidase and AT1R proteins in hD5R transgenic mice

On normal salt diet, the renal expression of Nox4 but not Nox2 (data not shown) was higher in hD5^{F173L} than hD5^{WT} transgenic mice (Figure 4a). On high salt diet the renal expression of Nox4 (but not Nox 2, data not shown) was also higher in hD5^{F173L} (Figure 4b) than hD5^{WT} transgenic mice (data not shown). In addition, renal AT1R expression was greater in hD5^{F173L} than hD5^{WT} transgenic mice on a normal salt diet (Figure 4c). However, AT1R protein expression was not affected by high salt diet in either strain (data not shown).

NADPH oxidase activity in hD5R transgenic mice

Consistent with the increased renal Nox4 expression in hD5^{F173L} mice (Figure 4a), renal NADPH oxidase activity was also increased in hD5^{F173L} relative to hD5^{WT} mice on a normal salt diet (Figure 5). High salt intake increased renal NADPH oxidase activity in both hD5^{F173L} and hD5^{WT} mice but it remained greater in hD5^{F173L} than hD5^{WT} transgenic mice (Figure 5). The dietary salt-induced increase in renal NADPH oxidase activity was decreased by candesartan treatment in hD5^{F173L} transgenic mice but was not affected in hD5^{WT} transgenic mice (Figure 5).

DISCUSSION

Dopamine, via its receptors, including the D5R, has been shown as an important regulator of blood pressure.^{2,8–11,16,17,23,24} In this study, we found that the blood pressures were increased in transgenic mice with the hD5R mutant, hD5^{F173L}, which had almost no ability to increase cAMP production in response to agonist stimulation. The hD5^{F173L} mice shows markedly increased Na/K ATPase levels and activities (Supplementary Data 1) and similar water and energy intake (Supplementary Data 2) compared with in hD5^{WT} mice on normal salt diet. A high salt intake increased further the blood pressure in hD5^{F173L} but had no effect in hD5^{WT} transgenic mice, which demonstrated the disorders of sodium excretion and salt sensitivity in hD5^{F173L} mice. We have reported that the blood pressures of transgenic mice expressing the D5R mutant hD5^{S390G}, which had an increase in cAMP production in response to agonist stimulation, were similar to those measured in hD5^{WT}

transgenic mice (data not shown). The current study reconfirmed the importance of D5R in the regulation of blood pressure and salt sensitivity.^{10,11,23}

It has been reported that oxidative stress is involved in the development of salt-sensitive hypertension.^{25–27} *In vivo* animal studies have demonstrated that increased ROS production in the renal cortex²⁸ and medulla²⁷ has an important role in the pathogenesis of salt-sensitive hypertension. In both human and experimental salt-sensitive hypertension, ROS production is increased and antioxidant capacity is decreased.^{26,27} ROS can inactivate the renal and vascular NO pathway, resulting in increased renal sodium transport²⁸ and afferent arteriolar resistance,²⁹ caused by impaired endothelium-dependent and -independent vasorelaxation.³⁰ ROS have also been shown to directly upregulate ion channels, transporters and pumps.³¹ These effects could lead to a decrease in sodium excretion, resulting in volume expansion and subsequently, hypertension.

We had reported that the renal expression and activity of NADPH oxidase were increased in D5^{-/-} mice relative to with D5^{WT} mice, and that the D5R agonist (in the absence of D1R) fenoldopam inhibited the superoxide production in HEK-293 cells transfected with hD5^{WT}.¹¹ In this study, in CHO cells transfected with the mutant hD5^{F173L}, superoxide production was increased and cAMP production was decreased relative to CHO cells transfected with hD5^{WT}. Nox4 expression and NADPH oxidase activity were greater in hD5^{F173L} mice than hD5^{WT} transgenic mice on normal salt diet. High salt diet increased the Nox4 expression in hD5^{F173L} but not in hD5^{WT} transgenic mice. High salt diet also increased NADPH oxidase activity in both hD5^{F173L} and hD5^{WT} transgenic mice but blood pressure was increased further in hD5^{F173L} but not in hD5^{WT} transgenic mice. High salt intake has been reported to increase ROS generation accompanied by enhanced renal NADPH oxidase expression and activity^{32,33} and blood pressure in predisposed animals, for example, salt-sensitive rats.^{25–27} In the current study, the increased NADPH oxidase activity (which presumably increased ROS production)¹¹ induced by high salt intake was unable to increase blood pressure in hD5^{WT} transgenic mice. This could be taken to indicate that the D5R may mitigate the production of ROS by increasing the activity of anti-oxidants, including heme-oxygenase 1,³⁴ paraoxonase 2³⁵ and superoxide dismutase,³⁶ among others. Moreover, the D5R can interact with other dopamine receptor subtypes to limit the production of ROS.³⁶

The renin–angiotensin system is paramount in the regulation of blood pressure.^{37,38} *In vitro* and *in vivo* studies have demonstrated that angiotensin II, via AT1R, stimulated the activity and expression of NADPH oxidase to increase the production of ROS.^{38–41} Candesartan, an AT1R antagonist, decreased renal NADPH oxidase activity in hD5^{F173L} mice fed a high salt diet but the effect that was not observed in hD5^{WT} mice. Thus hD5^{F173L} may interact with renin–angiotensin system to increase ROS production. Renal D5R and AT1R not only counter regulate each other's effect on sodium excretion but also their expression.^{16,22,23} In the current study, the renal AT1R expression was higher in hD5^{F173L} than hD5^{WT} transgenic mice fed normal salt diet. Although renal AT1R expression was no longer different between hD5^{F173L} than D5^{WT} mice, AT1R blockade normalized blood pressure in hD5^{F173L} mice on high salt diet, but had no effect on the normal blood pressure of hD5^{WT} transgenic mice. This could be taken to indicate that on high salt diet AT1R function is increased even though renal AT1R expression was not increased in D5^{F173L} mice. Dopamine

and its receptors and angiotensin II, via AT1R, have been shown to counter regulate each other in the regulation of renal tubule signaling, sodium transport, blood pressure and renal injury kidney.^{22,23,42–45} Thus a proper balance between dopamine and the renin–angiotensin systems in the kidney is needed to maintain normal renal sodium excretion and their abnormal interaction leads to sodium retention and ultimately an increase in blood pressure.

In conclusion, we have shown that the hD5R mutant, hD5^{F173L} increases ROS production and blood pressure that is, in part, related to an impaired ability to negatively regulate AT1R function. The expression of the hD5R variant, hD5^{F173L} in mice results in salt-sensitive hypertension and increased renal expression of AT1R similar to the germline deletion of D5R in mice.²³ However, the mechanisms involved in regulating the balance of renal D5R and AT1R in the oxidative stress-mediated salt-sensitive blood pressure remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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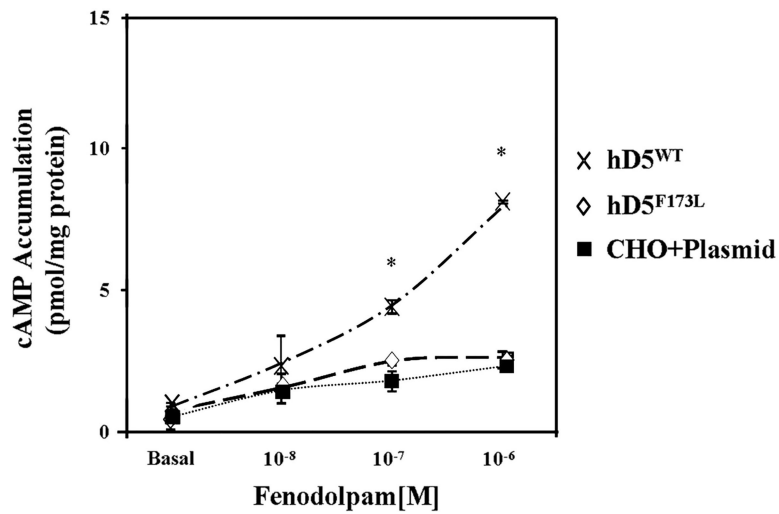


Figure 1.

cAMP accumulation in CHO cells. cAMP accumulation was determined in the presence of 1 mM 3-isobutyl-1-methylxanthine in hD5^{F173L}-, hD5^{WT}- and empty vector-transfected CHO cells treated with a D₁-like receptor agonist, fenoldopam (10⁻⁶ M, 30 min) ($n=3$ /group, mean \pm s.e., in some, error bars are smaller than the symbols, * $P<0.05$, one-way analysis of variance, Holm–Sidak test). In the absence of D1R, fenoldopam is a D5R agonist.

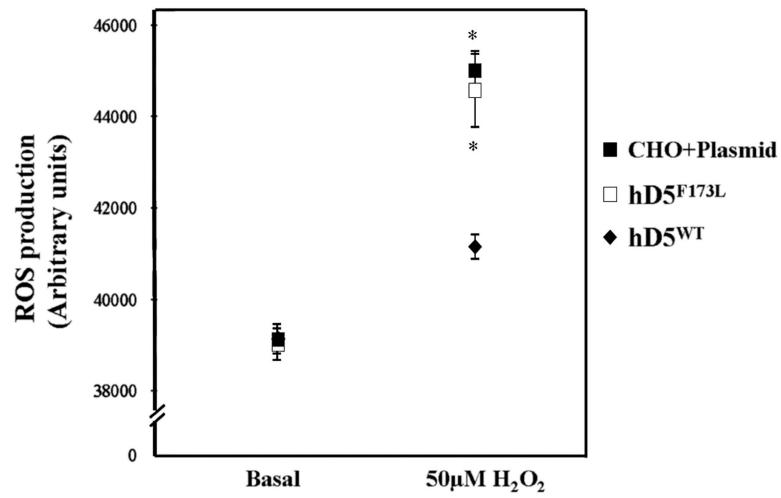


Figure 2. ROS production in CHO cells. The ROS production in hD5^{F173L}-, hD5^{WT}- and empty vector-transfected CHO cells treated with or without H₂O₂ (50 µM per 2 h) was measured by 2',7'-dichlorodihydrofluorescein diacetate ($n=3/\text{group}$, mean \pm SE, * $P<0.05$, one-way analysis of variance, Holm-Sidak test).

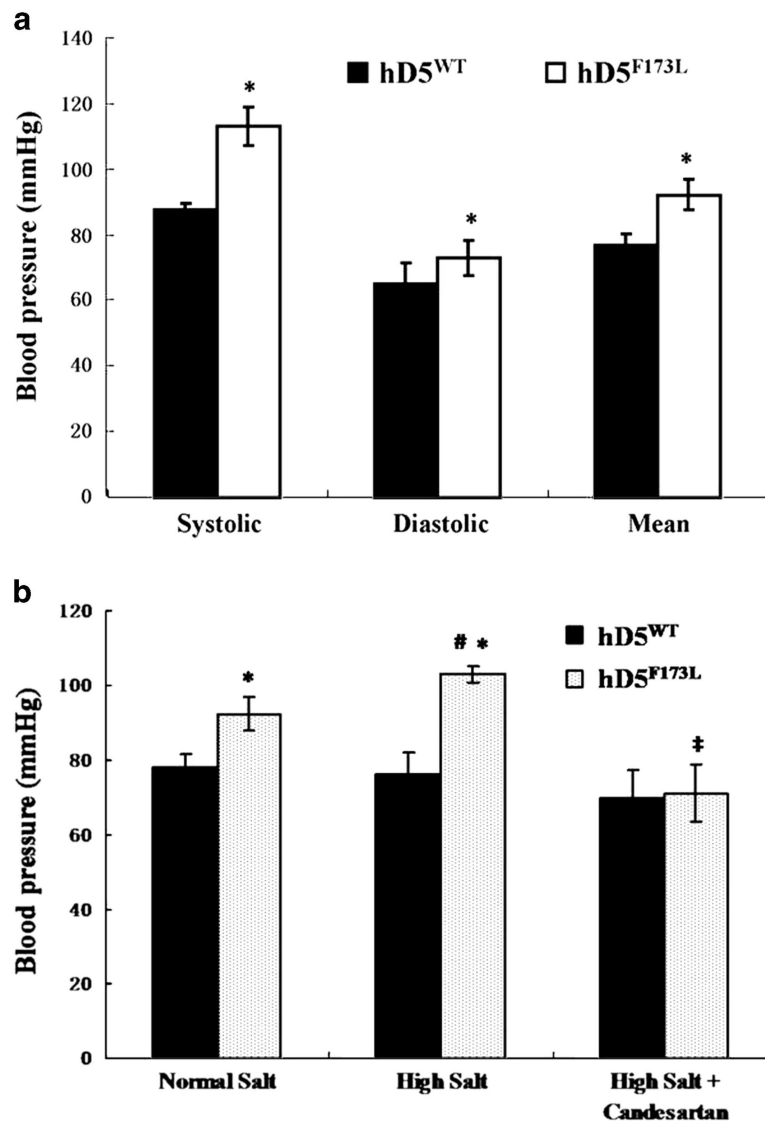


Figure 3. Arterial blood pressure in hD5^{WT} and hD5^{F173L} transgenic mice. (a) Systolic, diastolic and mean arterial blood pressures (measured under pentobarbital anesthesia) in hD5^{WT} and hD5^{F173L} transgenic mice on normal salt diet (0.8% NaCl). ($n=6$, $*P<0.05$ vs. hD5^{WT}, t -test). (b) Mean arterial blood pressure in hD5^{WT} and hD5^{F173L} mice fed normal salt (0.8% NaCl) or high salt diet (4% NaCl) for 14 and 15 days, respectively, and then treated with candesartan (1 mg kg⁻¹ body weight per day \times 10 days) ($n=6$, $*P<0.05$ vs. hD5^{WT}, t -test; # $P<0.05$ vs. others, two-way analysis of variance; ‡ $P<0.05$ vs. hD5^{F173L}-normal salt diet and hD5^{F173L}-high salt diet, two-way analysis of variance, Holm-Sidak test).

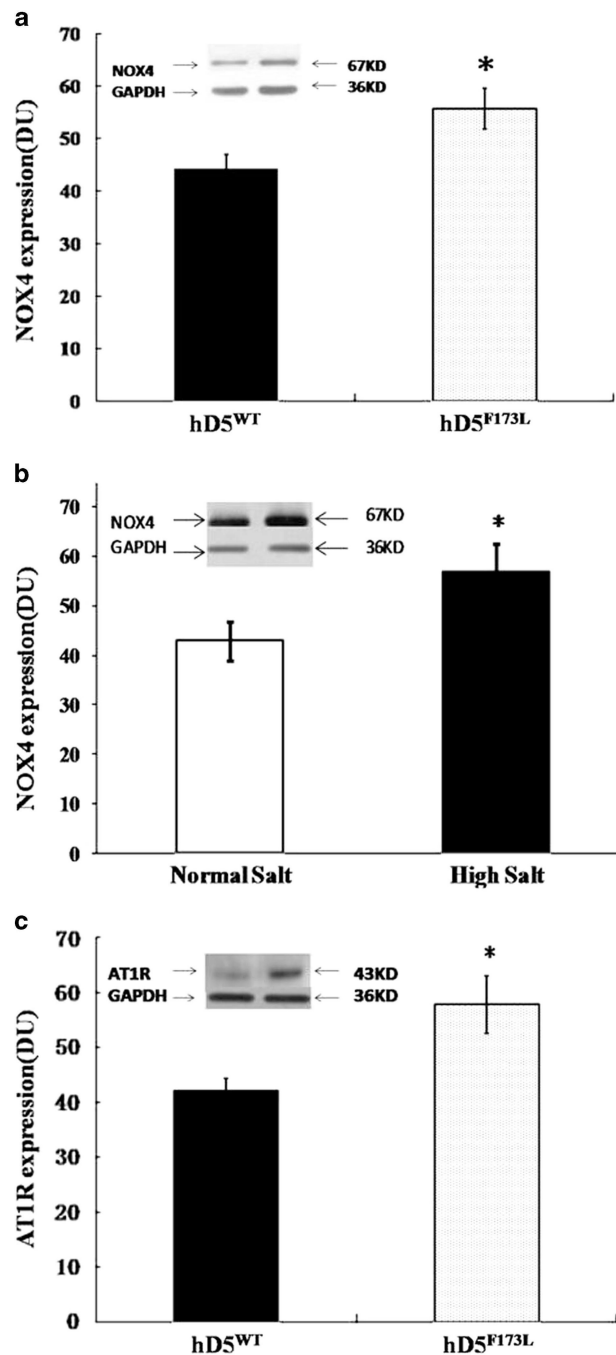


Figure 4. Renal NOX4 and AT1R protein expression in hD5^{WT} and hD5^{F173L} transgenic mice. (a) Renal NOX4 protein expression (67 kDa) determined by immunoblotting in hD5^{WT} and hD5^{F173L} transgenic mice fed normal salt diet ($n=6$, $*P<0.05$ vs. hD5^{WT}, t -test). (b) Renal NOX4 protein expression (67 kDa) determined by immunoblotting in hD5^{F173L} transgenic mice on normal or high salt diet ($n=6$, $*P<0.05$ vs. normal salt, t -test). (c) AT1R expression (43 kDa) determined by immunoblotting in hD5^{WT} and hD5^{F173L} mice on normal salt diet

(n=6, *P<0.05 vs. hD5^{WT}, *t*-test). All immunoblotting results are expressed as relative DUs. Immunoblots of NOX4, AT1R, and GAPDH are depicted in the inset. DU, density unit.

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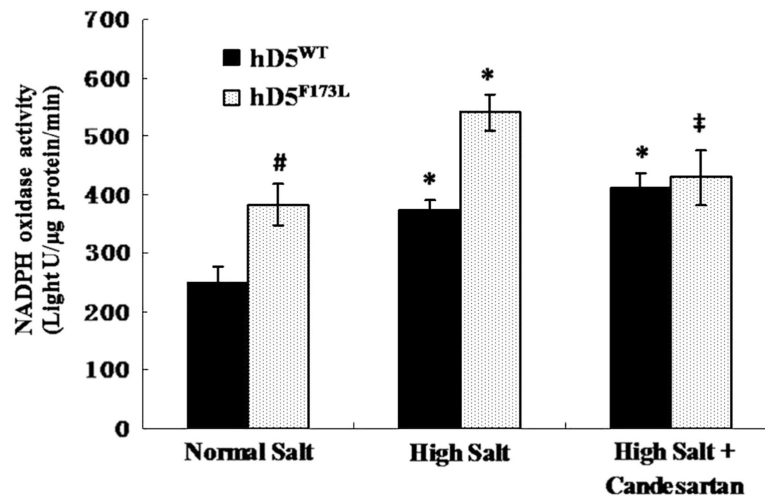


Figure 5. Renal NADPH oxidase activity in hD5^{WT} and hD5^{F173L} transgenic mice. NADPH oxidase activity, measured by lucigenin chemiluminescence, in kidney membranes from hD5^{WT} and hD5^{F173L} mice on a normal NaCl (0.8% NaCl) and high NaCl (4% NaCl) diets for 14 and 15 days, respectively, and treated with candesartan (1 mg kg⁻¹ body weight per day) for 10 days. Results are expressed as light units (U) per μg protein per min ($n=6$, $*P<0.05$ vs. normal salt; $^{\#}P<0.05$ vs. hD5^{WT} normal salt; $^{\ddagger}P<0.05$ vs. hD5^{F173L} high salt, t -test).