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Research Article

Since dopamine receptors are important in the regulation of renal and cardiovascular function, we studied the cardiovascular consequences of the disruption of the D3 receptor, a member of the family of D2-like receptors, expressed in renal proximal tubules and juxtaglomerular cells. Systolic and diastolic blood pressures were higher (approximately 20 mmHg) in heterozygous and homozygous than in wild-type mice. An acute saline load increased urine flow rate and sodium excretion to a similar extent in wild-type and heterozygous mice but the increase was attenuated in homozygous mice. Renal renin activity was much greater in homozygous than in wild-type mice; values for heterozygous mice were intermediate. Blockade of angiotensin II subtype-1 receptors decreased systolic blood pressure for a longer duration in mutant than in wild-type mice. Thus, disruption of the D3 receptor increases renal renin production and produces renal sodium retention and renin-dependent hypertension.

Disruption of the Dopamine D_3 Receptor Gene Produces Renin-dependent Hypertension

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Abstract

Since dopamine receptors are important in the regulation of renal and cardiovascular function, we studied the cardiovascular consequences of the disruption of the D_3 receptor, a member of the family of D_2-like receptors, expressed in renal proximal tubules and juxtaglomerular cells. Systolic and diastolic blood pressures were higher ($\sim$ 20 mmHg) in heterozygous and homozygous than in wild-type mice. An acute saline load increased urine flow rate and sodium excretion to a similar extent in wild-type and heterozygous mice but the increase was attenuated in homozygous mice. Renal renin activity was much greater in homozygous than in wild-type mice; values for heterozygous mice were intermediate. Blockade of angiotensin II subtype-1 receptors decreased systolic blood pressure for a longer duration in mutant than in wild-type mice. Thus, disruption of the D_3 receptor increases renal renin production and produces renal sodium retention and renin-dependent hypertension. (*J. Clin. Invest.* 1998. 102:493–498.) Key words: dopamine receptor • D_3 receptor gene • renin • catecholamines

Introduction

Essential hypertension affects 15–20% of the adult population and is one of the principal independent risk factors for stroke, myocardial infarction, and end-stage kidney disease (1). Except for some rare forms of monogenic hypertensive disease the cause(s) of essential hypertension is not known (1). The renin–angiotensin system has been implicated in the pathogenesis of hypertension, and individuals with essential hypertension have been categorized into those with low, normal, or high levels of plasma renin activity (2). The cause(s) of these differences in circulating renin activity is not known. No mutation of the genes associated with renin production has been found to be associated with essential hypertension, except for angiotensinogen (3). However, angiotensinogen polymorphism in essential hypertension is not associated with changes in plasma renin or angiotensin II levels (3, 4). More recently, a multicenter sib-pair analysis study failed to support a linkage or association between the human angiotensinogen locus and essential hypertension (5). Stimulation of a D_1-like receptor is associated with decreased renal sodium reabsorption, stimulation of renin release, and angiotensinogen gene expression (6–10). Thus, an abnormality resulting in inhibition of a D_1-like receptor function may lead to sodium retention and simultaneously produce a low renin state (10). In contrast, D_2-like receptors have been shown to inhibit renin release (11–13). The D_2-like receptor that negatively regulates renin secretion may be the D_3 receptor since it is expressed in juxtaglomerular cells while the D_{2Long} receptor is not (14). Quinpirole, a D_2-like agonist with preference for the D_3 and D_4 receptors over the D_2 receptor, decreases adenylyl cyclase activity (10, 14), which in turn decreases renin secretion. Z1046, a dopamine agonist with preference for the D_3 and D_4 receptor over the D_2, D_1, and D_5 receptors, inhibits renin secretion (15). The currently available animal models of high renin hypertension requires structural alteration to the kidney (renal artery stenosis) or the production of transgenic animals expressing increased copies of angiotensinogen or renin (16–18). Because the D_3 receptor is expressed in rat juxtaglomerular cells (14), we determined whether disruption of the D_3 receptor (mutant mice) results in abnormal blood pressure and if any abnormal blood pressure is related to the renin-angiotensin system (19). Mice mutant for the D_3 receptor gene demonstrate mild hyperactive behavior (19, 20) but their cardiovascular status has not been studied.

Methods

Generation of D_3 dopamine receptor mutant mice. Mice lacking the D_3 dopamine receptor (mutant mice) were generated by target mutagenesis. The targeting construct contained a 7-kb of 129/sv–derived D_3 dopamine receptor genomic sequence in the GKNeo cassette in antisense orientation at the SalI site in exon 2 (19). The gene was targeted in the J-1 line of ES cells (gift of R. Jaenisch, Whitehead Institute, Cambridge, MA). Homologous recombination resulted in a mutant allele in which sequences downstream of Arg-148 in the second intracellular loop of the D_3 receptor are replaced by sequences de-

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rived from the Neo gene. Homologous recombination events were identified by Southern blotting. Microinjection, transfer of 3.5-day postcoitus embryos, breeding of chimeric males with C57Bl/6 females, and generation of mice heterozygous and homozygous for the mutant gene were performed as reported (19).

Blood pressure and renal function studies in mice. The mice were anesthetized with pentobarbital (50 mg/kg intravenously) and tracheotomized (PE100; reference 21). Catheters were inserted into the femoral vessels (PE 50 heat-stretched to 180-μm tip) for fluid administration and blood pressure monitoring. Urine was collected via a suprapubic cystostomy (PE50-flanged end). After a 60-min stabilization period, a baseline 60-min period was obtained. Thereafter, a normal saline load equivalent to 5% of body weight was infused intravenously for 30 min. Urine was collected during the load (L)[1] and for another 30 min; two urine collection periods of 60 min each were obtained after the loading (post L1 and post L2). Blood (50 μl) was obtained from the femoral artery before the load and at the end of post L2. The kidneys were obtained for determination of renin, and D_3 mRNA and protein. The mice were killed with pentobarbital (100 mg/kg) at the end of the experiment.

Reverse transcriptase–PCR of D_3 receptors. Reverse transcriptase–PCR (RT-PCR) was performed using renal RNA and primers designed to amplify the wild-type D_3 receptor or mutant sequence (GKNeo insert; reference 19). Two sets of primers were used. One set was designed to amplify the wild-type gene: sense primer 5′-GCAGTGGTCATGCCAGTTCACTATCAG-3′ (nt 391–417) and antisense primer 5′-CCTGTTGTGTTGAAACCAAAGAGGAG-AGG-3′ (nt 526–498). The other set of primers was designed to amplify the mutant allele (KO): sense primer (nt 391–417) of the D_3 gene and antisense 5′-ATATTGCTGAAGAGCTTGGCG-3′ (GK-Neo). RT reaction was carried out for 15 min at an annealing temperature of 46°C and 42°C for the wild-type and mutant RNA respectively. PCR was performed using the following conditions: hot-start at 94°C for 5 min, denaturation at 92°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min for a total of 37 cycles for the wild-type and 32 cycles for the mutant gene. Approximately 4–6 μg of RNA were used during the RT step and all of the RT products were used for the subsequent PCR amplification. The sizes of the amplification products for the wild-type and the mutant cDNA were 135 and 600 bp, respectively.

Immunoblot of D_3 receptors. The antipeptide polyclonal IgG affinity-purified rabbit D_3 antibody was raised against a synthetic peptide sequence derived from the rat D_3 dopamine receptor. The specific sequence of the peptide was CHVSPELYR, amino acids 405–413, located on the third extracellular loop of the receptor (22). Immunoblotting used kidney homogenates mixed with Laemmli sample buffer, boiled for 5 min, and subjected to electrophoresis on 8% SDS-PAGE and then transferred electrophoretically to nitrocellulose membranes. The transblots were probed with the D_3 antibody (1:500) in Tris-HCl/saline/Tween-20 buffer for 2 h. Primary antibody binding was then probed by peroxidase-labeled anti–rabbit IgG donkey serum (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). D_3 specific bands were visualized using enhanced chemiluminescence (ECL; Western Blotting Detection Kit; Amersham Pharmacia Biotech, Inc.). HEK293 cells transfected with the rat D_3 cDNA were used for positive control. The immunoblots were quantified using Quantiscan (Biosoft, Ferguson, MO; reference 23).

Renin activity and catecholamines. Renin activity and angiotensin I (AI) concentrations were measured by RIA (24). The antibody used for AI has no cross-reactivity with other angiotensin peptides. The results for renin activity are reported as ng AI/ml/h, and for AI as pg/ml. The kidneys from mice studied were homogenized with 0.05 $MHClO_4$ /1 M $NaHSO_3$/0.1 M EDTA (2.8:0.1:0.1 ml), centrifuged at 6,000 *g* for 20 min at 4°C, and the supernatant was measured for norepinephrine by HPLC and electrochemical detection (24).

Statistical analyses. The data, which are expressed as mean± SEM, were analyzed by ANOVA for repeated measures when comparing within groups and one-way ANOVA when comparing among groups. $P < 0.05$ was considered significant.

Results

Arterial blood pressure in D_3 receptor mutant mice. In 3-mo-old F2 mice anesthetized with pentobarbital, systolic (SBP) and diastolic (DBP) blood pressures were higher in heterozygous (SBP = 117±3, DBP = 91±3 mmHg, n = 10) and homozygous (SBP = 120±2, DBP = 96±2 mmHg, n = 11) than in wild-type mice (SBP = 97±4, DBP = 79±4 mmHg, n = 9; Fig. 1). Body weights were similar among wild-type, heterozygous, and homozygous mice (wild-type = 33.8±4.0 g, heterozygote = 30.6±2.5 g, homozygote = 33.6±2.2 g).

D_3 receptor expression in D_3 mutant mice. The wild-type allele was present in wild-type mice and mice heterozygous for the D_3 receptor, but absent in the homozygous mice while the mutant allele was present in the heterozygous and homozygous mutant mice but not in the wild-type mice (Fig. 2 *A*). Protein expression was nearly absent in heterozygote mice in spite of the detection of the D_3 receptor mRNA of the wild-type allele in the heterozygote mice (Fig. 2 *B*), suggesting that the mutant D_3 receptor may have acted in a dominant-negative fashion to decrease expression of the normal allele (19).

Renal norepinephrine levels in D_3 mutant mice. Presynaptic D_3 receptors decrease the release of dopamine and norepi-

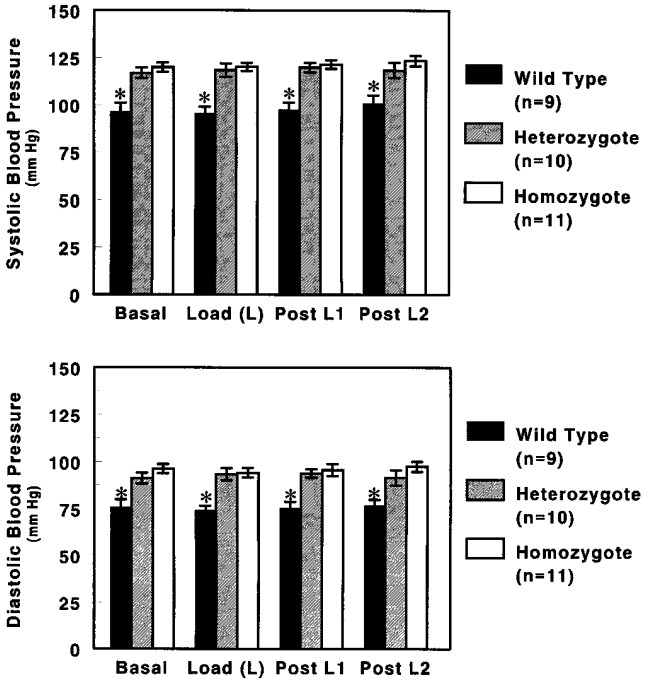

Figure 1. SBP and DBP in wild-type and mutant D_3 mice. The SBP and DBPs are higher in homozygous and heterozygous than in wild-type mice. An acute saline load does not affect either SBP or DBP. Data are expressed as mean±SEM. *$P < 0.05$ versus heterozygous or homozygous mice, one-way ANOVA, Scheffe's test.

1. *Abbreviations used in this paper:* AI, angiotensin I; DPB, diastolic blood pressure; L, load; RT-PCR, reverse transcriptase–PCR; SBP, systolic blood pressure.

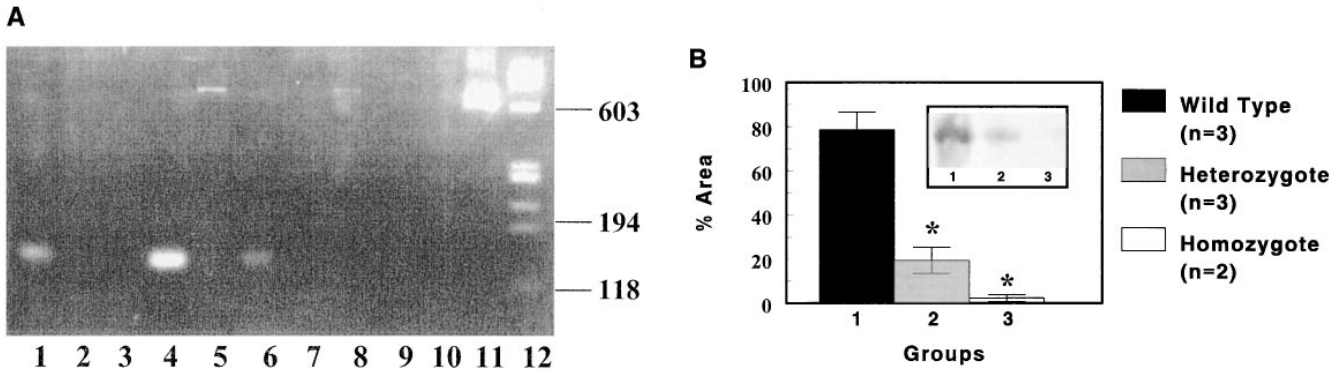

Figure 2. (*A*) Analysis of D_3 dopamine receptor RT-PCR products in 10% polyacrylamide gel stained with ethidium bromide. An amplification product of the predicted size (135 bp) is seen in RT-PCR reaction using RNA (4–6 μg) from kidneys of the D_3 wild-type mice and wild-type primers (lane *1*). No amplification is seen in the absence of RT (lane *2*) or when the primers for the mutant D_3 allele are used to amplify the wild-type allele (lane *3*). Lane *4* depicts the amplification product from genomic DNA (500 ng) of wild-type mice using wild-type primers. Lane *8* shows the amplification product of the predicted size (~ 600 bp) of the D_3 mutant allele using kidney RNA from homozygous mutant mice and primers for the D_3 mutant allele. No amplification product is seen in the absence of RT (lane *9*) or when the primers for the wild-type allele is used to amplify the mutant allele (lane *10*). Lane *11* depicts the amplification product from genomic DNA (500 ng) of mutant mice using primers for the mutant allele. Lanes *5* and *6* show the amplification products using RNA (4–6 μg) from kidneys of the D_3 heterozygous mice using primers for the mutant (lane *5*) and wild-type (lane *6*) allele, respectively. ϕX174 RFDNA/Haemophilus aegyptus ladder (bp) is seen in Lane *12*. Similar results were obtained in five other experiments. (*B*) Renal D_3 protein expression in wild-type and mutant D_3 mice. Renal D_3 receptor protein expression (50 kD) is absent in homozygous mice (lane *3*, *inset*) and markedly decreased in heterozygous mice (lane *2*, *inset*) compared with wild-type mice (lane *1*, *inset*). Kidneys were homogenized and prepared for immunoblotting (23). D_3 receptor protein was detected using anti-rat D_3 receptor antibodies (22). The intensity of the band was markedly decreased when the antibody was pre-adsorbed with the immunizing peptide; a 50-kD signal was noted in membranes from HEK 293 cells transfected with D_3 dopamine receptor cDNA (data not shown). Percent area denotes the relative density of the signal in each lane expressed as a fraction of 100%.

nephrine from nerve terminals (25). A possible consequence of the mutation is an increase in central and/or peripheral sympathetic activity that would then cause increased tissue catecholamine levels. However, renal norepinephrine levels were not different among the groups (Fig. 3). These results indicate that the D_3 receptor is not an important presynaptic receptor regulating norepinephrine release, at least in the mouse kidney.

Renal renin activity in D_3 receptor mutant mice. D_2-like dopamine receptors may negatively regulate renin secretion (13, 14). Because the D_3 but not the D_2 receptor is expressed in rat juxtaglomerular cells (14), we determined renal renin activity in D_3 receptor mutant mice. Fig. 4 shows that renal renin activity was highest in the homozygous mice, intermediate in the heterozygous mice, and least in the wild-type mice.

Angiotensin II type receptor blockade and arterial blood pressure in D_3 receptor mutant mice. To determine the functional relevance of increased renin production to arterial blood pressure, we measured the effect of a bolus intravenous injection of the angiotensin II subtype-1 receptor antagonist, losartan, on arterial blood pressure in anesthetized mice. The higher SBP in homozygous and heterozygous mutants compared with wild-type mice was again noted. The bolus intrave-

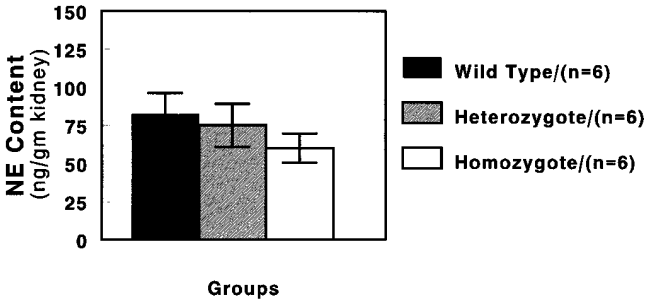

Figure 3. Renal norepinephrine levels in D_3 mutant mice. Renal norepinephrine concentration was measured in the kidneys of mice studied in Fig. 1. Renal norepinephrine concentrations were not different among the groups.

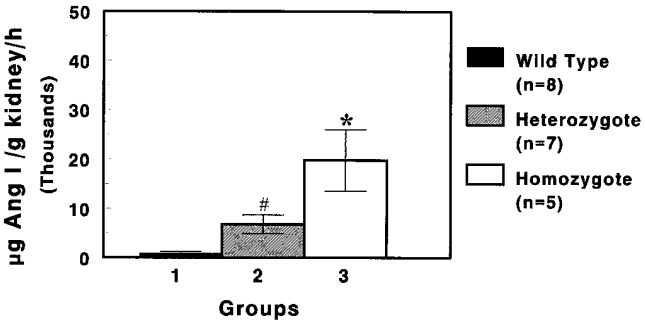

Figure 4. Renal renin activity in wild-type and mutant D_3 mice. Renal renin activity is highest in homozygous mice (lane *3*) compared with heterozygous (lane *2*) or wild-type mice (lane *1*). The kidneys were from mice studied in Fig. 1 and renin content was determined by angiotensin I generation and RIA (24). Data are expressed as mean±SEM. $^{*}P < 0.05$ versus others, ANOVA, Scheffe's test. $^{\#}P < 0.05$ versus wild type, ANOVA, Scheffe's test.

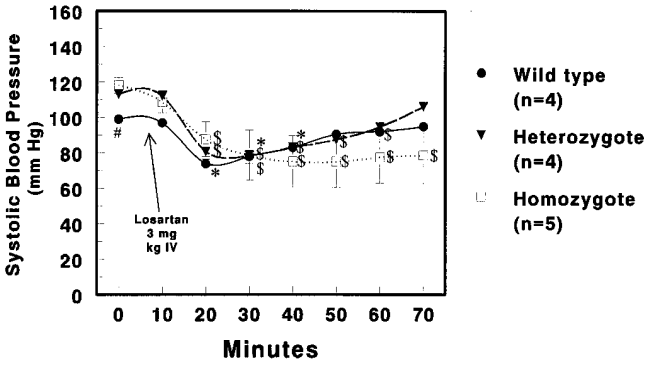

Figure 5. Effect of the angiotensin II type 1 receptor antagonist, losartan, on SBP in wild-type and mutant D_3 mice. A bolus intravenous administration of losartan (3 mg/kg), produced a more sustained decrease in SBP in heterozygous and homozygous than in wild-type mice. The mice were anesthetized with pentobarbital and the femoral artery catheterized as described in Fig. 1, but did not receive a saline load. Blood pressures were obtained after a 1-h stabilization period. Error bars are not evident when the symbol is bigger than the SEM. Data are expressed as mean±SEM. $^{*}P < 0.05$ versus 0 time, wild-type mice, ANVR, Scheffe's test; $^{\#}P < 0.05$ versus mutant mice, ANOVA, Scheffe's test; $^{\$}P < 0.05$ versus 0 time, mutant mice, ANVR, Scheffe's test.

nous injection of losartan (3 mg/kg body weight) decreased blood pressure to a similar degree in all groups of mice. In wild-type mice, SBP fell sharply within 10 min and recovered to baseline preinjection values 40 min after the bolus injection. In contrast, however, in heterozygous mice and homozygous mice the SBP remained depressed for as long as 50 and 60 min, respectively, after the bolus injection of the angiotensin II antagonist (Fig. 5).

Urine flow and sodium excretion in D_3 receptor mutant mice. The inhibitory effect of dopamine on renal sodium transport in rats involves a synergism between D_1- and D_2-like receptors (15, 26–28). Therefore, next we studied the cardiovascular consequences of an acute intravenous saline load equivalent to 5% of the body weight. The acute saline load did not affect mean arterial blood pressure. Sodium excretion was significantly less in homozygous than in heterozygous mice in the last period after saline loading (Fig. 6). Urine flow after saline loading tended to be greatest in wild-type mice, intermediate in the heterozygous mice, and least in the homozygous mice, but the differences did not achieve statistical significance (data not shown).

Discussion

Our studies show that disruption of the D_3 receptor, a member of the family of D_2-like receptors, increases SBP and produces diastolic hypertension in heterozygous and homozygous mice. The increased blood pressure is associated with increased renal renin activity. Dopamine and dopaminergic drugs have been reported to increase renin secretion in some studies and to decrease it in other studies (8, 11–13). These apparently conflicting reports can now be explained by the contrasting effects on renin secretion after stimulation of dopamine receptor subtypes. Rat juxtaglomerular cells are innervated by dopaminergic nerves and express the D_{1A} and D_3 receptor subtypes (8, 14, 29, 30). D_1-like receptors, probably via the D_{1A} receptor subtype in the rat, are responsible for the dopamine-mediated increase in renin secretion (6–8). The other D_1-like receptor, the D_{1B} receptor, is not expressed in rat juxtaglomerular cells (8). D_2-like receptors probably mediate the inhibitory effect of dopamine on renin secretion (11–13). Since D_3 but not D_2 receptors are present in rat juxtaglomerular cells and since D_3 receptor stimulation inhibits cAMP accumulation in these cells, we have suggested that the D_3 receptor may be responsible for the dopamine-mediated decrease in renin secretion in these cells (14, 15). In these studies we show that D_3 receptors are functionally important negative modulators of renal renin secretion. Which effect of dopamine or dopaminergic drugs on renin secretion prevails depends not only on the selectivity of the drug to the dopamine receptor subtype but also on the state of the extracellular fluid volume. The ability of dopamine to stimulate renin secretion is enhanced by a low sodium diet and blunted by a high sodium diet (31).

The increased renal renin activity in the D_3 receptor mutant mice may be a cause of the high blood pressure in both homozygous and heterozygous mice since losartan decreased systemic blood pressure to a longer extent in the mutant than in the wild-type mice. Why the blood pressure in homozygous mice is not higher than in heterozygous mice remains to be determined. Previous studies in the brain and our studies in the kidney show that D_3 receptor protein expression was nearly absent in heterozygote mice in spite of the detection of the D_3

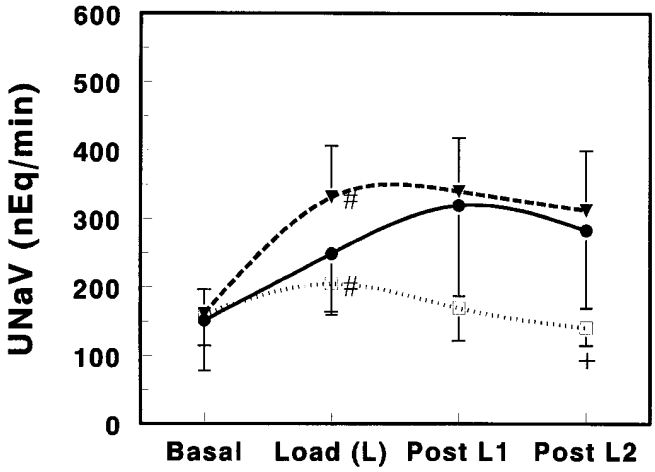

Figure 6. Effect of an acute saline load (5% body weight) on sodium excretion in wild-type and mutant D_3 mice studied in Fig. 1. There was a mild increase in sodium excretion immediately after the load in the mutant mice. Sodium excretion was less in the homozygote than the heterozygote mice at Post L2. For abbreviations and methods, see Fig. 1. Data are expressed as mean±SEM. $^{\#}P < 0.05$ versus Basal, *t* pair, Bonferroni correction; $^{+}P = 0.06$ versus heterozygote mice, *t* test.

receptor mRNA of the wild-type allele in the heterozygote mice, suggesting that the mutant D_3 receptor may have acted in a dominant-negative fashion to decrease expression of the normal allele (19). However, this may not explain the effects on renal renin activity since renin activity was greater in homozygous than heterozygous mice. It is possible that there is a threshold effect of the renin-angiotensin system on blood pressure, that compensatory mechanisms become more fully operative when there is a complete abrogation of D_3 receptor expression or that the phenotype is modified with age as a result of an interaction between genetic and environmental factors (1). Nevertheless, the elevated blood pressure and the increase in renal renin activity in heterozygote mice suggest that the differences between the mutant and the wild-type mice could not be ascribed to heterogeneity of genetic background (32). The increased renin production probably contributed to the increase in arterial blood pressure since the bolus intravenous injection of the angiotensin II subtype-1 receptor antagonist, losartan, on arterial blood pressure decreased blood pressure. Although the magnitude of blood pressure decrease seemed to be greatest in the homozygous mice, the changes were not statistically significant. However, the duration of the hypotensive effect of losartan was longest in the homozygous mice and shortest in the wild-type mice. These data support the hypothesis that the increase in blood pressure in mutant mice is renin dependent. These results constitute the first example of a gene (other than genes directly involved in renin secretion such as angiotensinogen) that controls renin secretion, disruption of which leads to an increased renal renin levels and elevated blood pressure. Mice lacking endothelial nitric oxide synthase have both elevated blood pressure and plasma renin levels but renal renin levels are actually lower in homozygous than in wild-type mice (33). The investigators concluded that this paradox of elevated plasma renin and low renal renin concentration in mutant mice could have been related to the blood withdrawal procedure. The effect of the elevated plasma renin on blood pressure in mice lacking endothelial nitric oxide synthase was not tested (33).

Presynaptic D_2-like receptors decrease the release of dopamine and norepinephrine from nerve terminals (25). A decreased ability of dopamine to inhibit norepinephrine release in mesenteric arteries has been reported in the spontaneously hypertensive rat (34). A lack of presynaptic function in the D_3 receptor mutant mice could have increased renal nerve activity, resulting in an increase in tissue catecholamine levels. However, renal norepinephrine levels were not different among the groups. These results indicate that the D_3 receptor is not an important presynaptic receptor regulating norepinephrine release, at least in the mouse kidney. In the brain, the D_3 receptor has not been found to be significantly involved in dopamine autoreceptor function either (25).

Dopamine has been shown to act as an intrarenal natriuretic hormone in rodents and humans. The natriuretic effect of dopamine, which becomes manifest only in sodium replete states, is mediated mainly by dopamine D_1-like receptors (35). Uncoupling of a dopamine D_1-like receptor from its G-protein/effector enzyme complex in renal proximal tubules results in a decreased ability to excrete a sodium load; in the SHR this abnormality cosegregates with hypertension (10, 21, 35–37). Moreover, disruption of the D_{1A} receptor produces hypertension in mice (21), an effect that may be caused by a decreased ability to excrete a sodium load. However, the inhibitory effect of dopamine on renal sodium transport in rats involves a synergism between D_1-like and D_2-like receptors (26–28). An acute saline load did not affect mean arterial blood pressure in wild-type or mutant mice. The tendency for stratification of urine flow among the groups was not significant but urine–sodium excretion was significantly less in homozygous than in heterozygous mice. A decreased ability to excrete a sodium load could contribute to the high blood pressure found in the homozygous mice. Some of the genetically determined phenotypes associated with essential hypertension have been ascribed to impaired ability of the kidney to excrete a sodium after chronic loading (38). The inability of D_1-like receptors to affect normal sodium excretion in this setting may be due to a need for a complementary D_2-like action. It has been reported that a synergistic action between D_1- and D_2-like receptors is needed to inhibit Na^+/K^+ ATPase activity in renal proximal tubules (26, 27). The relevance between this study and the increased expression of the D_3 receptor in human essential hypertension remains to be determined (39).

In summary, this is the first report of a model of high renin hypertension that does not involve structural damage to the kidney or manipulation of genes that are directly involved in angiotensin II production. Studies on these mice may prove to be important in the understanding of high renin essential hypertension. Whether a naturally occurring mutation of the dopamine D_3 receptor gene is present in genetic hypertension remains to be determined.

Acknowledgments

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