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ARTICLE

Antihypertensive effect of etamicastat in dopamine D2 receptor-deficient mice

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Abstract

Abnormalities of the D_2R gene (*DRD2*) play a role in the pathogenesis of human essential hypertension; variants of the *DRD2* have been reported to be associated with hypertension. Disruption of *Drd2* ($D_2^{-/-}$) in mice increases blood pressure. The hypertension of $D_2^{-/-}$ mice has been related, in part, to increased sympathetic activity, renal oxidative stress, and renal endothelin B receptor (ETBR) expression. We tested in $D_2^{-/-}$ mice the effect of etamicastat, a reversible peripheral inhibitor of dopamine-β-hydroxylase that reduces the biosynthesis of norepinephrine from dopamine and decreases sympathetic nerve activity. Blood pressure was measured in anesthetized $D_2^{-/-}$ mice treated with etamicastat by gavage, (10 mg/kg), conscious $D_2^{-/-}$ mice, and $D_2^{+/+}$ littermates, and mice with the D_2R selectively silenced in the kidney, treated with etamicastat in the drinking water (10 mg/kg per day). Tissue and urinary catecholamines and renal expression of selected G protein-coupled receptors, enzymes related to the production of reactive oxygen species, and sodium transporters were also measured. Etamicastat decreased blood pressure both in anesthetized and conscious $D_2^{-/-}$ mice and mice with renal-selective silencing of D_2R to levels similar or close to those measured in $D_2^{+/+}$ littermates. Etamicastat decreased cardiac and renal norepinephrine and increased cardiac and urinary dopamine levels in $D_2^{-/-}$ mice. It also normalized the increased renal protein expressions of ETBR, NADPH oxidase isoenzymes, and urinary 8-isoprostane, as well as renal NHE3 and NCC, and increased the renal expression of D_1R but not D_5R in $D_2^{-/-}$ mice. In conclusion, etamicastat is effective in normalizing the increased blood pressure and some of the abnormal renal biochemical alterations of $D_2^{-/-}$ mice.

Introduction

Inhibition of dopamine β-hydroxylase (DBH) may provide significant clinical improvement in patients suffering from cardiovascular disorders, such as hypertension and chronic heart failure. The rationale for the use of DBH inhibitors is based on their ability to inhibit the biosynthesis of norepinephrine (NE), via inhibition of the enzymatic hydroxylation of dopamine (DA) [1].

Direct inhibition of sympathetic nerve function by reducing the biosynthesis of NE, preventing the conversion of DA to NE in sympathetic nerves, and possibly by increasing the release of DA, can induce renal vasodilation, diuresis, and natriuresis. β-adrenergic blockers are no longer recommended as primary therapy for hypertension except for patients with coexisting conditions, such as coronary heart disease or left ventricular dysfunction. α-adrenergic blockers are also not recommended as first-line therapy for hypertension because they may be associated with increased incidence of adverse cerebrovascular and cardiovascular outcomes [2]. Therefore, inhibitors of DBH may provide significant clinical advantages over other drug treatments, especially those that affect the sympathetic nervous system.

Etamicastat [(R)-5-(2-aminoethyl)-1-(6,8-difluorochroman-3-yl)-1,3-dihydroimidazole-2-thione hydrochloride] is a potent, reversible inhibitor of peripheral DBH with limited

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access to the brain [1]. In spontaneously hypertensive rats (SHRs) but not in normotensive control rats, oral administration of etamicastat lowered both systolic and diastolic blood pressures in a dose-dependent manner without affecting the heart rate [3]. Etamicastat, chronically administered in drinking water, also significantly reduced both blood pressure and urinary NE excretion but increased urinary DA excretion in the SHR [4]. This DBH inhibitor has also been shown to decrease blood pressure in hypertensive patients [5].

The intrarenal dopaminergic system plays an important role in the normal regulation of renal sodium excretion and blood pressure [6, 7]. Human essential hypertension and some rodent models of genetic hypertension are associated with decreased renal DA production and receptor function [6, 8]. Both DA D2-like (*Drd2*, *Drd3*, and *Drd4*) and D1-like (*Drd1* and *Drd5*) receptors have been shown to regulate arterial blood pressure. Abnormalities of the D_2R gene (*DRD2*) play a role in the pathogenesis of human essential hypertension; several variants of the human *DRD2* have been reported to be associated with hypertension [9, 10] and disruption ($D_2^{-/-}$) or renal-selective silencing of *Drd2* in mice increases systolic and diastolic blood pressures [11–13] and may cause salt sensitivity [14]. The hypertension of $D_2^{-/-}$ mice has been related to increased sympathetic and vascular smooth muscle endothelin B receptor (ETBR) activities [11], as well as increased reactive oxygen species (ROS) [12].

In this study, we determined the effects of inhibition of DBH and NE formation with etamicastat on blood pressure in $D_2^{-/-}$ mice. We measured blood pressure in conscious and anesthetized $D_2^{-/-}$ mice and $D_2^{+/+}$ littermates after acute- and short-term administration of etamicastat, catecholamine levels in the heart, kidney, and urine, and renal expression of selected G protein-coupled receptor and enzymes related to and a parameter of ROS production. In addition, we studied the effect of etamicastat on the elevated blood pressure of mice in which renal cortical *Drd2* was silenced by the renal subcapsular infusion of *Drd2*-specific small interfering RNA (siRNA), via an osmotic minipump [13]. We also determined the expression of selected sodium transporters, exchangers, and channels in the kidney of $D_2^{-/-}$ mice and $D_2^{+/+}$ littermates before and after treatment with etamicastat.

Materials and methods

D_2 receptor-deficient mice

The original F2 hybrid strain (129/SvXC57Bl/6J, Oregon Health Sciences University, Portland) that contained the mutated D_2R allele ($D_2^{-/-}$) was backcrossed into wild-type C57Bl/6J for >20 generations and genotyped [10]. All mice were bred in the Animal Care Facility of the University of Maryland School of Medicine and The George Washington University School of Medicine & Health Sciences. Male $D_2^{-/-}$ mice and $D_2^{+/+}$ littermates fed 0.6% NaCl were studied at 4–6 months of age. All studies were approved by the Animal Care and Use Committees of the University of Maryland School of Medicine and The George Washington University School of Medicine & Health Sciences.

Treatment with etamicastat and blood pressure measurements

Acute treatment

Etamicastat (10 mg/kg), synthesized in the Department of Chemistry, BIAL - Portela & C[a], S.A. Portugal, with a purity of 99.5%, or vehicle (tap water) was administered by gastric gavage (200 µL) to $D_2^{-/-}$ and $D_2^{+/+}$ mice. The mice were individually housed in metabolic cages for collection of urine samples before measurement of blood pressure. Blood pressure was measured 9 or 18 h after drug administration. Systolic and diastolic blood pressures were measured (Cardiomax II, Instruments, Columbus, OH) from the aorta, via the femoral artery, under pentobarbital anesthesia (50 mg/kg, administered intraperitoneally). Blood pressures were recorded 1 h after the induction of anesthesia when blood pressures were stable. The mice were killed (pentobarbital 100 mg/kg) at the conclusion of the study. The hearts and kidneys were collected, frozen in isopentane at −30 °C on dry ice, and stored at −80 °C until studied. Tissue and urine catecholamines were quantified, as reported [1, 15, 16].

Short-term etamicastat treatment in conscious mice

TA-PAC20 transmitters (Data Sciences International, St. Paul, MN) were implanted into the carotid artery of $D_2^{-/-}$ and $D_2^{+/+}$ mice under isoflurane anesthesia, and blood pressures were measured on individual platforms 1 week after the surgery [17, 18]. Etamicastat (10 mg/kg per day) or vehicle (tap water) was added in the drinking water after baseline blood pressure measurement. The mice were monitored for 5 days after starting drug treatment. Blood pressure and heart rate were recorded every 10 min throughout the study. Data were collected and stored automatically in a dedicated computer that ran and analyzed the data (Dataquest). Thereafter, the mice were killed, kidneys were collected and the renal expression of selected G protein-coupled receptors, ROS-related enzymes, and selected sodium transporters, exchangers, and channels were quantified, as reported [11, 12, 17–23].

In another study, mice implanted with TA-PAC20 transmitters were treated with etamicastat (10 mg/kg per day in the drinking water) for 5 days and fed a normal salt (0.6% NaCl) diet. At the end of this period, the dose of etamicastat was increased to 50 mg/kg per day for another 5 days. Blood pressure was recorded (one reading per h) on the last day on each treatment.

Acute renal-specific downregulation of D_2R

Renal cortical *Drd2* was silenced by the renal subcapsular infusion of *Drd2*-specific siRNA, via an osmotic minipump [13, 19, 20]. Adult male C57BL/6J mice were uninephrectomized 1 week before the implantation of the minipump. Osmotic minipumps (ALZET® Osmotic Pump, 100 µl; flow rate 0.5 µl/h for 7 days) were filled with previously validated *Drd2*-specific siRNA (delivery rate 3 µg per day) or non-silencing siRNA as control. The siRNAs were dissolved in an in vivo transfection reagent (TransIT® In Vivo Gene Delivery System, Mirus) under sterile conditions. The minipumps were fitted with a polyethylene delivery tubing (Alzet #0007701) and the tip of the tubing was inserted within the subcapsular space of the remaining kidney. Etamicastat treatment (10 mg/kg per day in the drinking water) was started immediately after pump implantation. Blood pressure was measured under pentobarbital anesthesia, as described, 7 days after pump implantation.

Immunoblotting

Whole-kidney lysates were prepared in lysis buffer supplemented with protease inhibitors, as previously reported [11, 12, 17–23]. Samples with equal amounts of proteins were separated by 10% SDS-polyacrylamide gel (Bio-Rad) electrophoresis and transferred onto nitrocellulose membranes. The membranes were sequentially probed with the primary antibodies (1:5000) at 4 °C overnight and corresponding horseradish peroxidase-conjugated secondary antibodies (1:10,000, Pierce at room temperature for 1 h). Chemiluminescence was detected using SuperSignal West Dura Substrate (Thermo Fisher Scientific, Waltham, MA), followed by autoradiography. The band densities of the proteins of interest were quantified by the NIH Image J and normalized by corresponding total actin bands. Alternatively, for infrared detection of protein signal, the membranes were probed with Infra-red dye 680- or 800-labeled secondary antibodies (Li-COR Bioscience, Lincoln, NE). The band densities of the proteins of interest were quantified using the Odyssey Infrared imaging system (Li-COR) and normalized by corresponding total actin bands.

The rabbit polyclonal antibodies against DA receptors D_1R (DRD1), D_3R (DRD3), D_4R (DRD4), and D_5R (DRD5) were generated in our laboratory while rabbit polyclonal antibodies against D_2R (EMD Millipore, Billerica, MA) and actin (Sigma-Aldrich, St. Louis, MO) were purchased. We have reported the specificity of our D_1R antibody [18, 20], D_3R antibody [19], D_4R antibody [17], D_5R antibody [21, 22], D_1R antibody from Origene (Rockville, MD) [22], and D_2R from EMD Millipore [13]. The sources of other antibodies used were: NHE3, NKCC2, NCC, αENaC, βENaC, γENac, generous gifts of Dr. Mark Knepper [21]; ETBR (Alomone Labs, Jerusalem, Israel); NOX1 (NADPH Oxidase 1, Santa Cruz Biotechnologies, Dallas, TX), NOX2 (gp91 phox, Upstate Biotech/Thermo Fisher Scientific), and HO-1 (Enzo Life Sciences, Farmingdale, NY).

Assay of catecholamines

Catecholamines in urine and tissue (DA and NE) were assayed by high-performance liquid chromatography with electrochemical detection, as previously described. The lower limit of detection of DA and NE is 350 fmol [1, 15].

Assay of 8-isoprostane

Urinary isoprostane, a parameter of oxidative stress, was measured by enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI) [12]. Values were corrected for urinary creatinine.

Statistical analysis

Data are reported as mean ± SEM. Comparisons between two groups used the Student's *t* test. One-way analysis of variance, followed by Holm–Sidak test, was used to assess significant differences among three or more groups. $P < 0.05$ was considered statistically significant.

Results

Etamicastat decreases blood pressure in mice with germ line deletion of D_2R or renal-silenced D_2R

Systolic blood pressure measured under anesthesia was higher in $D_2^{-/-}$ mice than in $D_2^{+/+}$ littermates treated with vehicle (122 ± 1 vs. 96 ± 6 mmHg, $n = 4$–5 per group). Systolic blood pressure also measured under anesthesia, 9 or 18 h after gavage administration of 10 mg/kg etamicastat, was decreased in $D_2^{-/-}$ mice to levels similar to those in $D_2^{+/+}$ littermates (Fig. 1a). Diastolic blood pressures, which were increased in $D_2^{-/-}$ mice relative to their $D_2^{+/+}$ littermates (91 ± 1 vs 68 ± 2 mmHg; $P < 0.05$), were normalized at 9 h ($D_2^{-/-}$: 72 ± 5 mmHg; $D_2^{+/+}$: 72 ± 1 mmHg) and 18 h

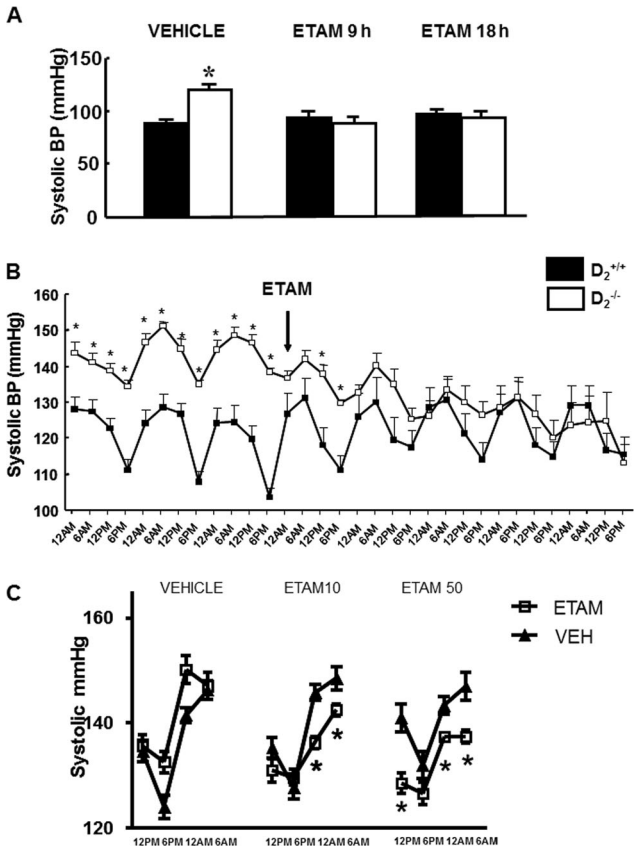

Fig. 1 Effect of etamicastat on blood pressure in anesthetized and conscious $D_2^{+/+}$ and $D_2^{-/-}$ mice. **a** Etamicastat (ETAM, 10 mg/kg) or vehicle was administered by gavage. Blood pressure was measured under pentobarbital anesthesia 9 and 18 h after etamicastat administration in different groups of $D_2^{+/+}$ and $D_2^{-/-}$ mice. $n = 4$–5 per group; $^*P < 0.05$ vs all others, one-way ANOVA followed by Holm–Sidak test. **b** Etamicastat (ETAM, 10 mg/kg per day) or vehicle was added to the drinking water. Blood pressure was measured by telemetry in conscious mice. Values are means of 6 h measurements (one per h). $D_2^{+/+}$ $n = 5$; $D_2^{-/-}$ $n = 4$. $^*P < 0.05$ vs $D_2^{+/+}$ one-way ANOVA followed by Holm–Sidak test. **c** Etamicastat (ETAM, 10 or 50 mg/kg per day) was added to the drinking water. Blood pressure was measured by telemetry in conscious mice. Values shown are means of 6 h measurements during the day and night (one reading per h after 5 days on each treatment). $D_2^{+/+}$ $n = 4$; $D_2^{-/-}$ $n = 3$. $^*P < 0.05$ vs $D_2^{-/-}$ vehicle or same treatment $D_2^{+/+}$, two-way ANOVA followed by Holm–Sidak test

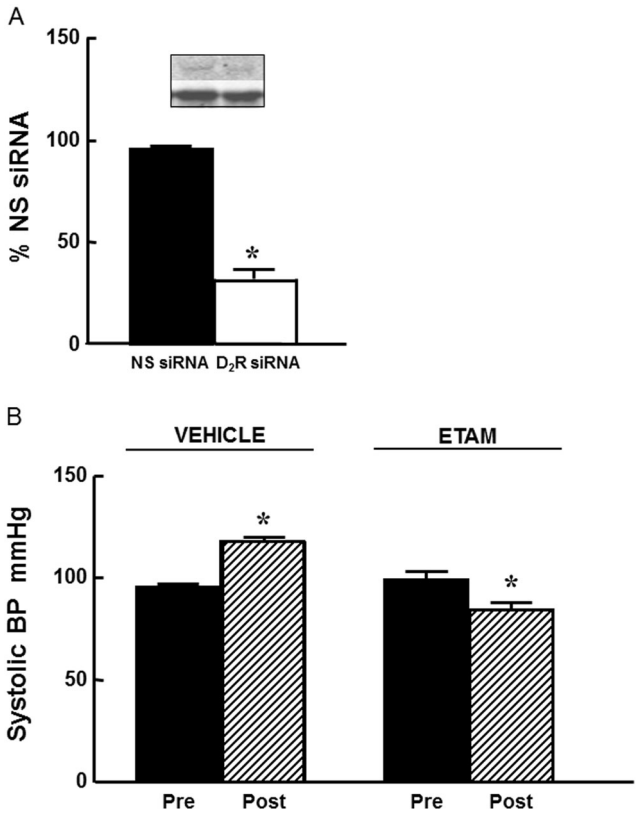

Fig. 2 Effect of etamicastat on blood pressure in mice with renal-selective silencing of the D_2R. **a** Renal cortical expression of D_2R in the remnant kidney of adult male uninephrectomized C57Bl/6J mice treated with a renal subcapsular infusion of *Drd2* siRNA or non-silencing (NS) siRNA for 7 days ($n = 6$ per group; $P < 0.05$, Student's *t* test). **b** Renal cortical *Drd2* was silenced by the renal subcapsular infusion of *Drd2* siRNA, via an osmotic minipump for 7 days in uninephrectomized adult male C57Bl/6J mice (Methods). Etamicastat (ETAM, 10 mg/kg per day in the drinking water) treatment was started immediately after pump implantation. Blood pressure was measured under anesthesia before and 7 days after pump implantation. $n = 5$ per group; $^*P < 0.05$ vs all others; one-way ANOVA followed by Holm–Sidak test. Two-way ANOVA positive for effect of D_2R siRNA and etamicastat

($D_2^{-/-}$: 75 ± 3 mmHg; $D_2^{+/+}$: 76 ± 4 mmHg) after the administration of etamicastat.

Systolic blood pressure measured by telemetry in conscious mice was also higher in $D_2^{-/-}$ than $D_2^{+/+}$ mice (Fig. 1b). Administration of etamicastat (10 mg/kg per day, $n = 4$–5 per group), added to the drinking water, also decreased systolic blood pressure in $D_2^{-/-}$ mice but had no significant effect in $D_2^{+/+}$ littermates. The decrease in systolic blood pressure was noted 24 h after starting treatment and persisted throughout the duration of the study (Fig. 1b). The decrease in systolic blood pressure was more marked during the night when the mice are awake, feeding, and drinking water (Fig. 1c). The conscious systolic blood pressure during the day or night was similar in mice treated for 5 days with a 10 or 50 mg/kg per day dose of etamicastat.

Renal cortical-selective silencing of the D_2R increased blood pressure in mice [13]. As shown in Fig. 2a, in mice implanted with an osmotic minipump for the continuous renal subcapsular infusion of D_2R siRNA, renal D_2R expression was decreased to about 70% as we have reported previously [13]. These mice have increased systolic blood pressure under anesthesia. The increase in blood pressure resulting from the D_2R siRNA infusion was prevented in mice treated with etamicastat in the drinking water (10 mg/kg per day, $n = 5$ per group) during the 7 days of D_2R siRNA infusion (Fig. 2b).

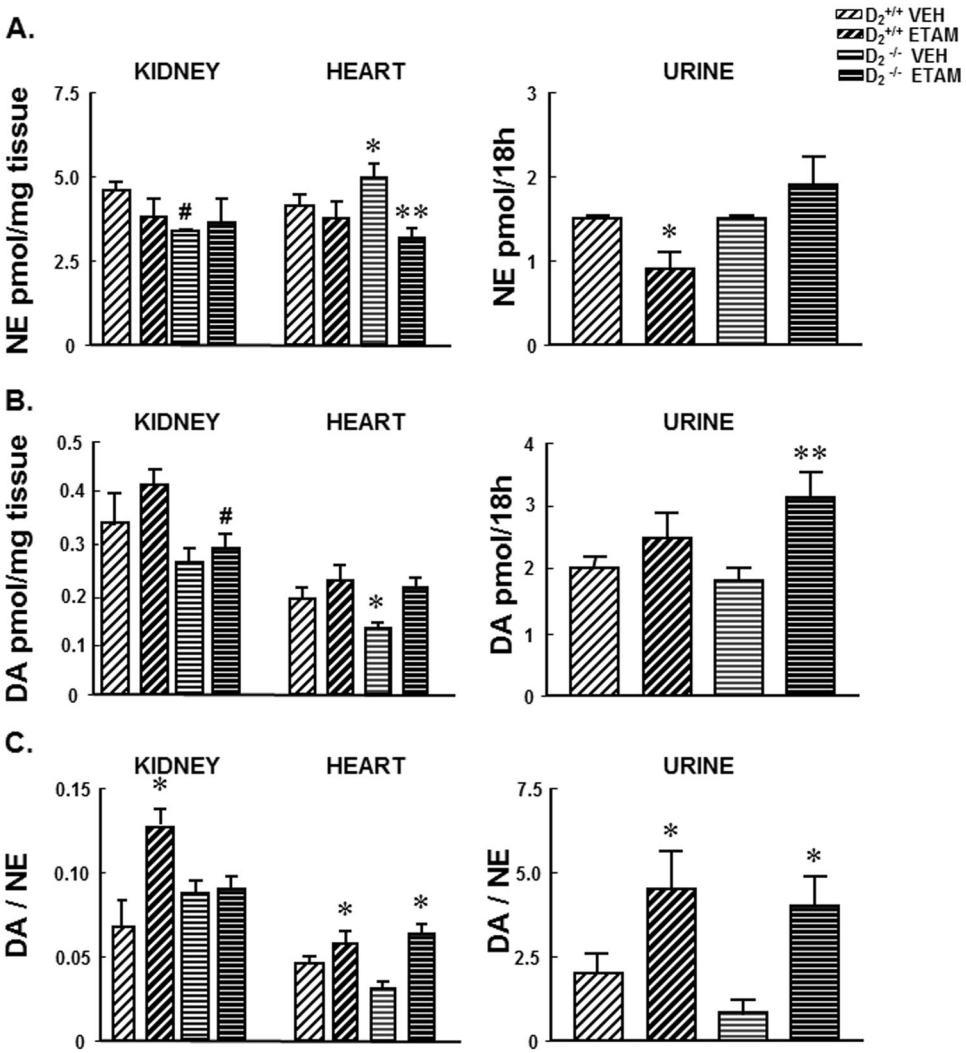

Fig. 3 Effect of etamicastat on norepinephrine (NE) and dopamine (DA) content in kidney, heart, and urine of $D_2^{+/+}$ and $D_2^{-/-}$ mice. Etamicastat (ETAM, 10 mg/kg per dose) or vehicle was administered by gavage. Urine collection was started immediately after gavage and lasted for 18 h. Tissues were collected after ending the urine collection. Urine and tissue NE (**a**) and DA (**b**) were measured by HPLC-ED, $n = 5–8$ per group. The tissue or urine DA/NE ratio (**c**) was calculated. $^*P < 0.05$ vs all others; $^{**}P < 0.05$ vs same group vehicle-treated mice; #P<0.05 vs $D_2^{+/+}$ same treatment; one-way ANOVA followed by Holm–Sidak test

Effect of etamicastat on catecholamines in tissue and urine

The cardiac NE content was higher in vehicle-treated $D_2^{-/-}$ than $D_2^{+/+}$ mice. Eighteen hours after the stomach gavage of etamicastat, cardiac NE content decreased in $D_2^{-/-}$ mice but was minimally affected in $D_2^{+/+}$ mice. Renal NE content was higher in vehicle-treated $D_2^{+/+}$ than in $D_2^{-/-}$ mice. Eighteen hours after the acute administration, etamicastat had no significant effect on renal NE content in $D_2^{+/+}$ or $D_2^{-/-}$ mice. By contrast, urinary excretion of NE was similar in vehicle-treated $D_2^{+/+}$ and $D_2^{-/-}$ mice; etamicastat treatment decreased NE excretion significantly in $D_2^{+/+}$ mice only (Fig. 3a, $n = 5–8$ per group).

The cardiac DA content, which was lower in vehicle-treated $D_2^{-/-}$ than $D_2^{+/+}$, was increased by etamicastat in $D_2^{-/-}$ but not in $D_2^{+/+}$ mice. Renal DA content was similar in vehicle-treated $D_2^{+/+}$ and $D_2^{-/-}$ mice and was not significantly changed by treatment with etamicastat. However, renal DA content after etamicastat was lower in $D_2^{-/-}$ than $D_2^{+/+}$ mice. Urinary DA was similar in untreated mice of both strains but was increased by etamicastat in $D_2^{-/-}$ but not $D_2^{+/+}$ mice (Fig. 3b). The DA/NE ratio was lower, although not significantly, in tissues and urine of untreated $D_2^{-/-}$ than in $D_2^{+/+}$ mice and increased by etamicastat in the heart and urine of both $D_2^{+/+}$ and $D_2^{-/-}$ mice; in the kidney, etamicastat increased the DA/NE ratio only in $D_2^{+/+}$ mice (Fig. 3c).

Effect of etamicastat on the renal expression of dopamine and endothelin B receptors

In $D_2^{-/-}$ mice, relative to $D_2^{+/+}$ littermates, the renal protein expressions of D_3R and ETBR were increased. Etamicastat treatment did not alter the increased renal expression of D_3R, but increased D_1R expression, normalized ETBR expression, and decreased D_5R expression (Fig. 4a, b, $n =$ 4–5 per group).

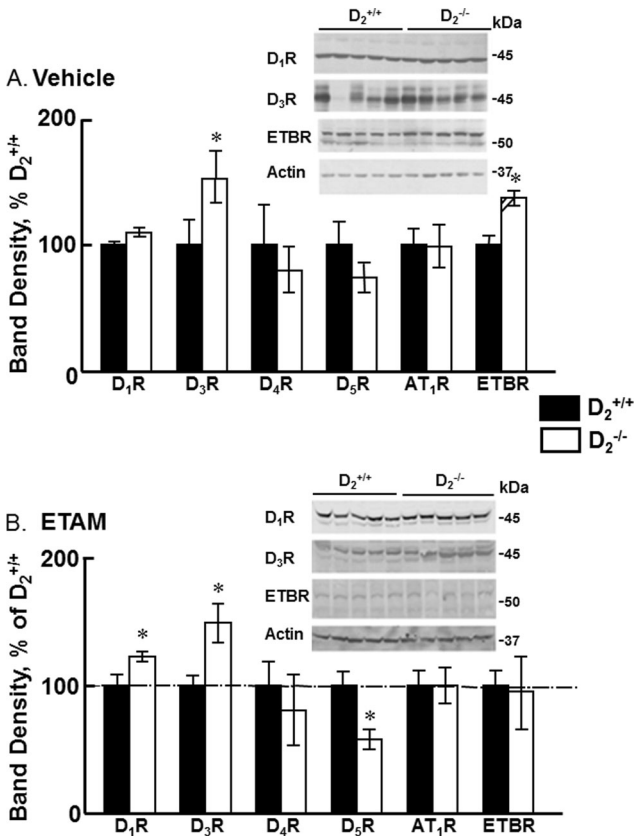

Fig. 4 Effect of etamicastat on the renal expression of dopamine, angiotensin II type 1, and endothelin B receptors. Etamicastat (ETAM, 10 mg/kg per day) was added to the drinking water for 5 days. Receptor expression was determined by immunoblotting. **a** Renal receptor expression in vehicle-treated mice; $n = 8$–10 per group; (**b**) renal receptor expression in etamicastat-treated mice; $n = 4$–5 per group. *$P < 0.05$ vs $D_2^{+/+}$ mice; Student's t-test

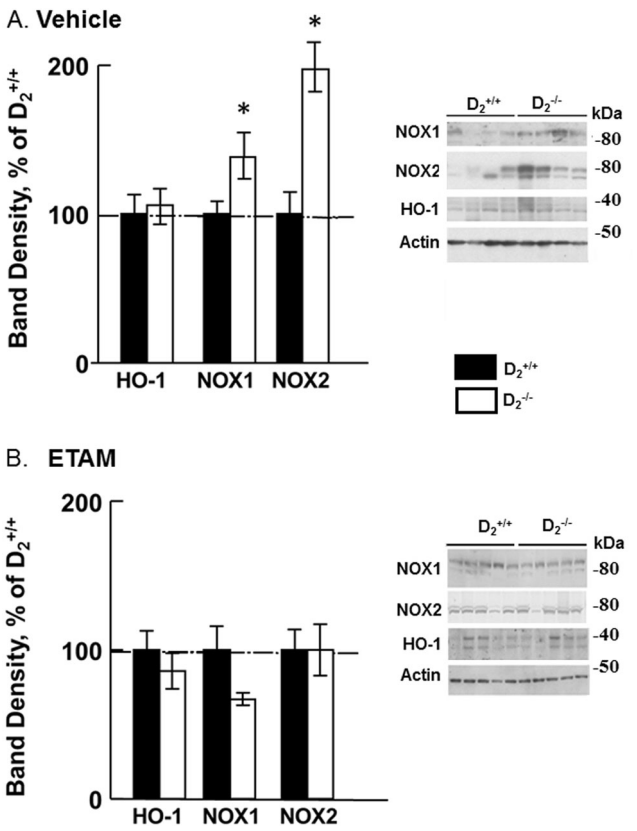

Fig. 5 Effect of etamicastat on the renal expression of ROS-related enzymes. Etamicastat (ETAM, 10 mg/kg per day) was added to the drinking water for 5 days. Expression of HO-1, NOX1, and NOX2, was determined by immunoblotting. **a** Expression of renal HO-1 and NOX isoforms in vehicle-treated mice; $n = 5$ per group; (**b**) expression of renal HO-1 and NOX isoforms in etamicastat-treated mice; $n = 5$ per group. *$P < 0.05$ vs $D_2^{+/+}$ mice; Student's t-test

Effect of etamicastat on the renal expression of ROS-related enzymes

In agreement with our previous report [12], the renal expressions of NADPH oxidase isoforms NOX1 and NOX2 were increased in $D_2^{-/-}$ mice, relative to $D_2^{+/+}$ littermates. Treatment with etamicastat normalized the expression of these NOX isoforms but did not affect HO-1 expression in $D_2^{-/-}$ mice (Fig. 5a, b, $n = 4$–5 per group). Moreover, the urinary excretion of isoprostane, a product of the nonenzymatic oxidation of arachidonic acid, and a marker of oxidative stress, was increased in vehicle-treated $D_2^{-/-}$ mice and was almost completely normalized by etamicastat treatment (Fig. 6; $n = 4$ per group).

Effect of etamicastat on the expression of selected renal sodium cotransporters, exchangers, and channels in $D_2^{-/-}$ mice

The renal protein expressions of sodium hydrogen exchanger type 3 (NHE3) and sodium chloride

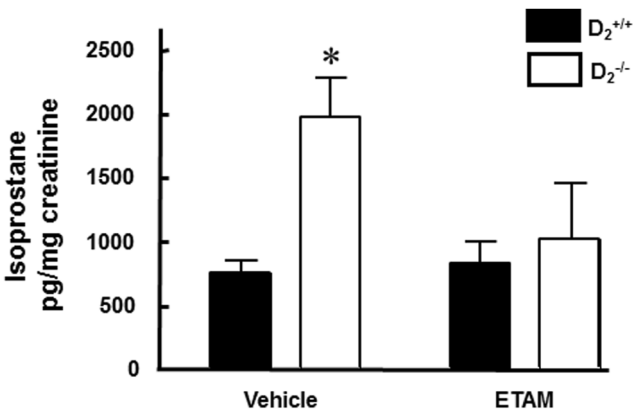

Fig. 6 Effect of etamiscastat on the urinary excretion of 8-isoprostane. Mice were studied before and after administration or etamicastat (ETAM, 10 mg/kg per day) added to the drinking water for 5 days. Urine was collected in metabolic cages. $n = 4$ per group, *$P < 0.05$ vs all others; one-way ANOVA followed by Holm–Sidak test

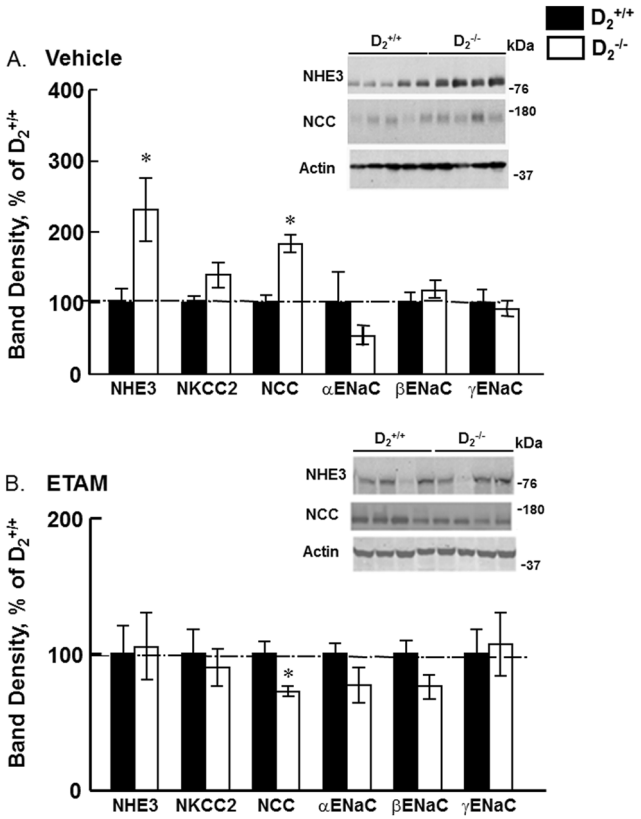

Fig. 7 Effect of etamicastat on the renal expression of sodium transporters, exchanger, and channels. Etamicastat (ETAM, 10 mg/kg per day) was added to the drinking water for 5 days. Renal expression of sodium transporters, exchanger, and channels was determined by immunoblotting. **a** Renal expression in vehicle-treated mice; $n = 4$–5 per group; (**b**) renal expression in ETAM-treated mice; $n = 4$ per group. $^{*}P < 0.05$ vs $D_2^{+/+}$ mice; Student's t-test

cotransporter (NCC) were increased in $D_2^{-/-}$ mice, relative to $D_2^{+/+}$ littermates. Etamicastat normalized the renal protein expression of NHE3 and decreased that of NCC (Fig. 7a, b, $n = 4$–5 per group).

Discussion

The results of this study show that either the acute- or short-term administration of the peripheral DBH inhibitor, etamicastat, decreases blood pressure in both non-anesthetized and anesthetized mice with germ line deletion of *Drd2* or renal-selective silencing of *Drd2* (renal subcapsular infusion of *Drd2* siRNA in $D_2^{+/+}$ mice) but not in wild-type littermates or $D_2^{+/+}$ mice that received renal subcapsular infusion of non-silencing siRNA. The reduction in blood pressure in $D_2^{-/-}$ mice caused by etamicastat correlates with an increase in the DA/NE content in heart and urine (but not kidney) and D_1R expression in the kidney, as well as a normalization of renal NOX isoforms (NOX1 and NOX2) and proximal tubule NHE3 and a decrease in distal convoluted tubule NCC. These could be taken to indicate that the increase in urinary DA does not reflect total renal DA but is specific to the nephron segment where DA is produced, i.e., renal proximal tubule. The DA produced in the renal proximal tubule is secreted in the tubular lumen affecting sodium transporters beyond the proximal tubule [6].

The reduction in blood pressure in hypertensive $D_2^{-/-}$ mice agrees with the reported ability of etamicastat to decrease blood pressure in hypertensive rats and humans [3–5]. The hypertension in $D_2^{-/-}$ mice is, in part, related to increased sympathetic activity proved by the ability of α1-adrenergic receptor blockade and adrenalectomy to decrease the elevated blood pressure of $D_2^{-/-}$ mice to the same level as that noted in $D_2^{+/+}$ littermates [11]. The two models we have used in this study are different in that one involves global germ line deletion of the D_2R while the other involves a 7-day renal subcapsular infusion of *Drd2* siRNA in the remaining kidney of uninephrectomized mice that decreases renal D_2R expression by 60–70%. Hemodynamic changes to compensate for the uninephrectomy may increase sympathetic nervous activity and potentiate the effect of D_2R silencing. In fact, we have reported that uninephrectomy plus silencing of D_2R in the remnant kidney increases blood pressure to the same extent as that observed with global deletion of the gene; the same period (1 week) of siRNA infusion in one kidney while the other kidney is unperturbed has no significant effect on blood pressure [13]. However, if the D_2R siRNA is infused for 28 days instead of 7 days, the blood pressure increases to the same extent as the global germ line deletion of D_2R [24]. These results point to a predominant role of the kidney in the hypertension that develops in mice with deficient D_2R function.

The NE content of the heart (but not kidney) was increased in vehicle-treated $D_2^{-/-}$ mice, which may reflect the increased sympathetic activity in these mice [11, 25]. Etamicastat treatment of these $D_2^{-/-}$ mice significantly decreased cardiac NE and increased cardiac DA. In wild-type littermates treated with etamicastat, there was a trend for NE to decrease and DA to increase, but the effects were not statistically significant. In previous studies using etamicastat, the decrease in DBH activity and sympathetic drive was associated with a decrease in NE in the heart of wild-type mice [1] and urine in healthy human subjects [26]. In the current study, cardiac NE content was decreased by 40% in $D_2^{-/-}$ mice but minimally in $D_2^{+/+}$, 18 h after administration of a 10 mg/kg dose of etamicastat. This modest decrease may be due to differences in sympathetic activity between $D_2^{-/-}$ and $D_2^{+/+}$mice [11, 27]. By contrast, it has been shown that, in normal mice, a 100 mg/kg dose of etamicastat decreased heart NE content by 75% [1].

Deletion of the *Dbh* gene in mice is associated with NE deficiency. However, the extracellular levels of DA are also decreased in the nucleus accumbens and caudate putamen but not in the prefrontal cortex and increased in adrenergic neurons, cerebellum, liver, lung, retina, skeletal muscle, and spleen of $Dbh^{-/-}$ mice on mixed C57BL/6 and 129/SvEv background [28, 29].

Urine NE and DA levels, were similar in $D_2^{-/-}$ and their wild-type littermates, in agreement with our previous report [11] and with the failure of others to find differences in DA levels in the brain striatum of $D_2^{-/-}$ and $D_2^{+/+}$ mice [30]. Basal DA efflux in the striatum was reported to be similar in $D_2^{-/-}$ and $D_2^{+/+}$ mice [30], although an increase in DA metabolites was found by others [31]. In the current study, etamicastat did not alter significantly renal NE content. In $D_2^{+/+}$ mice, etamicastat decreased urinary NE. Because about 35% of urinary NE is derived from the kidney [25], this result may suggest that the inhibitory effect of etamicastat is more pronounced or of longer duration in the heart than in the kidney. It is also possible that the failure of etamicastat to decrease NE content in the kidney may be related to the fact that DA produced by the renal proximal tubule is not converted to NE; renal proximal tubules do not express DBH (see below).

The increase in blood pressure in $D_2^{-/-}$ mice is not due to an increase in the activity of the renin–angiotensin system [11]. However, we have reported that the hypertension in $D_2^{-/-}$ mice is associated with increased production of ROS, accompanied by increased expression of NOX enzymes, results that were corroborated in the present study [12]. Treatment with etamicastat normalized the renal expression of NOX enzymes and almost normalized the levels of ROS, as determined by urinary levels of 8-isoprostane. In $D_2^{-/-}$ mice, treatment with spironolactone decreased blood pressure, and normalized the increased expression of NOX1 and NOX4 but had no effect on the increased NOX2. Thus, it is possible that the increase in blood pressure in $D_2^{-/-}$ mice drives the increase in NOX isoforms expression.

In tissues outside the central nervous system, the inhibition of DBH increases DA release [32, 33]; DA has vasorelaxant effects that cause an increase in renal blood flow and indirectly abets the increase in sodium excretion caused by direct inhibition of renal tubular sodium transport [6]. Independent of innervation, the kidney synthesizes DA that is not metabolized to NE and prevents the ability of moderate sodium load to increase blood pressure by inducing diuresis and natriuresis [6, 34]. Cardiac and urinary DA levels are increased in $D_2^{-/-}$ mice after etamicastat administration. In spite of the fact that DA produced by the kidney from L-DOPA is not metabolized to NE because DBH is not expressed in renal tubules [35], inhibition of DBH could still increase renal DA because of blockade of

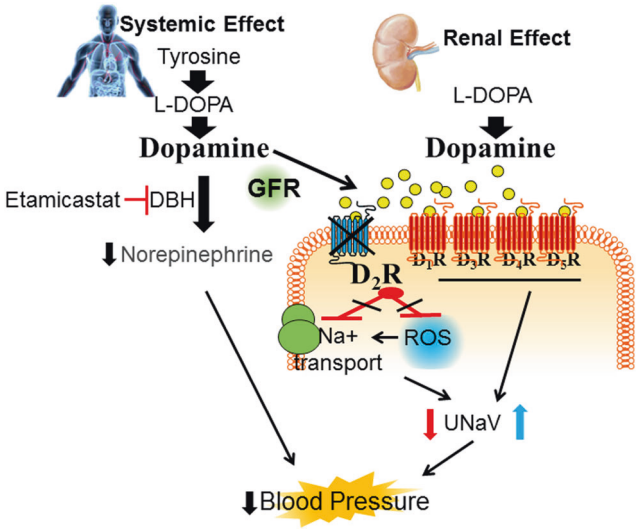

Fig. 8 Filtered dopamine, as well as dopamine produced in the renal proximal tubule, stimulates dopamine receptors to increase sodium excretion under conditions of normal or moderate sodium load. Deletion of any dopamine receptor gene increases blood pressure. Etamicastat inhibits dopamine β hydroxylase (DBH), decreasing the conversion of norepinephrine to dopamine, allowing the levels of dopamine to increase. The increase in filtered dopamine and dopamine produced by the kidney (presumably from the renal nerves; DBH is not expressed in renal tubules) stimulate the other dopamine receptor subtypes, D_1R, D_3R, D_4R, and D_5R, which overcomes the absence or decreased expression of D_2R. The increased production of reactive oxygen species (ROS) is normalized, sodium excretion (UNaV) is increased, and blood pressure is normalized in mice without D_2R in the body or mice with decreased renal D_2R expression

NE production from renal nerves [36]. Most if not all the DA in the urine is synthesized in the kidney (specifically in the proximal tubules) under normal conditions [6, 36, 37]. However, an increase in DA release in peripheral tissues, caused by inhibition of DBH by etamicastat may substantially increase DA in the circulation, and thus, in the glomerular filtrate (Fig. 8). The increase in the DA/NE ratio in the urine in both groups could also reflect an increase in renal production of DA. In fact, it has been reported that in patients with congenital DBH deficiency, not only is free plasma DA increased but also plasma DOPA that would increase the substrate for the synthesis of urinary DA [38].

DA increases renal sodium excretion, in part, by inhibiting renal NHE3, sodium phosphate cotransporters (NaPi-IIa, NaPi-IIc), Cl^-/HCO_3^- exchanger, sodium bicarbonate exchanger (NBCe1), NaKATPase, NCC, ENaC, and potassium channel [6]. Treatment with etamicastat reversed the increased renal protein expressions of NHE3, NCC, and ETBR protein in $D_2^{-/-}$ mice suggesting that these changes were secondary to either the increased sympathetic activity or blood pressure. However, etamicastat also increased renal D_1R expression, did not alter the increased D_3R expression and decreased D_5R expression in $D_2^{-/-}$ mice,

indicating some specific effect on renal DA receptor expression when renal/urinary DA is increased.

The human *DRD2* gene encoding the D_2R is highly polymorphic. The presence of some single-nucleotide polymorphisms (SNPs; rs6276, rs6277, and rs1800497) in the *DRD2* gene are associated with decreased D_2R expression and function attributable to decreased D_2R messenger RNA stability and synthesis of the receptor and are associated with elevated blood pressure and hypertension [10, 39]. These SNPs are highly prevalent with minor allele frequencies ranging from 0.24 to 0.47 [40]. From the pharmacogenomics point of view, etamicastat would be ideal to treat hypertensive patients carrying these SNPs.

In conclusion, etamicastat is effective in normalizing blood pressure in $D_2^{-/-}$ mice, in which hypertension is caused, in part, by increased activity of the sympathetic nervous system. In $D_2^{-/-}$ mice, etamicastat normalized the increased renal expression on NHE3 and decreased the renal expression of NCC. Etamicastat also increased the renal expression of D_1R and normalized the increased renal expression of ETBR, decreased the renal expression of D_5R without affecting the increased renal expression of D_3R. Etamicastat also normalized the increased renal expression of NOX1 and NOX2 isoenzymes. Whether or not these effects are primary or secondary to the etamicastat-induced decrease in blood pressure in $D_2^{-/-}$ mice remains to be determined. However, we have reported that decreasing the blood pressure of $D_2^{-/-}$ mice with spironolactone does not normalize the increased renal NOX2 expression in $D_2^{-/-}$ mice suggesting that the negative regulation of NOX expression by etamicastat may be blood pressure independent.

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Compliance with ethical standards

Conflict of interest P.S.S. is an employee of BIAL - Portela & C[a], S.A. (the sponsor of the study). The remaining authors declare that they have no conflict of interest.

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