ARTICLE

Acute salt loading induces sympathetic nervous system overdrive in mice lacking salt-inducible kinase 1 (SIK1)

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Abstract

Loss of salt-inducible kinase 1 (SIK1) triggers an increase in blood pressure (BP) upon a chronic high-salt intake in mice. Here, we further addressed the possible early mechanisms that may relate to the observed rise in BP in mice lacking SIK1. SIK1 knockout ($sik1^{-/-}$) and wild-type ($sik1^{+/+}$) littermate mice were challenged with either a high-salt (8% NaCl) or control (0.3% NaCl) diet for 7 days. Systolic BP was significantly increased in $sik1^{-/-}$ mice after 7 days of high-salt diet as compared with $sik1^{+/+}$ mice and to $sik1^{-/-}$ counterparts on a control diet. The renin–angiotensin–aldosterone system and the sympathetic nervous system were assayed to investigate possible causes for the increase in BP in $sik1^{-/-}$ mice fed a 7-day high-salt diet. Although no differences in serum renin and angiotensin II levels were observed, a reduction in aldosterone serum levels was observed in mice fed a high-salt diet. Urinary L-DOPA and noradrenaline levels were significantly increased in $sik1^{-/-}$ mice fed a high-salt diet as compared with $sik1^{-/-}$ mice on a control diet. Similarly, the activity of dopamine β-hydroxylase (DβH), the enzyme that converts dopamine to noradrenaline, was significantly increased in the adrenal glands of $sik1^{-/-}$ mice on a high-salt intake compared with $sik1^{+/+}$ and $sik1^{-/-}$ mice on a control diet. Treatment with etamicastat (50 mg/kg/day), a peripheral reversible DβH inhibitor, administered prior to high-salt diet, completely prevented the systolic BP increase in $sik1^{-/-}$ mice. In conclusion, SIK1 activity is necessary to prevent the development of salt-induced high blood pressure and associated SNS overactivity.

Keywords SIK1 · High-salt intake · Sympathetic nervous system · Dopamine β-hydroxylase · Hypertension

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Introduction

Salt-inducible kinase 1 (SIK1) is a sucrose non-fermenting-like kinase isoform that belongs to the AMP-activated protein kinase (AMPK) family of serine/threonine kinases [1]. SIK1 is part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process [2]. SIK1 activity is increased by high-salt intake and is implicated in regulation of the plasma membrane Na^+, K^+-ATPase (NKA) activity [3]. SIK1 regulates active sodium transport in the renal and lung epithelia by increasing NKA activity and mediates gene expression activation in cardiac myocytes upon increase in intracellular sodium [2, 4, 5]. Variations in intracellular sodium concentrations upon increases in cell sodium permeability triggers the activation of plasma membrane NKA activity in order to maintain cellular homeostasis. Consequently, the lack of SIK1 is associated with reduction in NKA activity and protein [6, 7].

SIK1 is also localised in human vascular smooth muscle cells (VSMCs) and endothelial cells, and its activity appears

to be of potential relevance for VSMCs function and blood pressure regulation [8]. A nonsynonymous single-nucleotide polymorphism in the hSIK1 gene exon 3 results in the amino acid change (15)Gly → Ser in the SIK1 protein, which is associated with lower blood pressure and with a decrease in left ventricular mass [8]. Moreover, SIK1 is also present in the brain, and it has been proposed that dysregulation of the SIK1–NKA network in neurons contributes to salt-induced hypertension through angiotensin-mediated sympathetic hyperactivity by increasing intracellular $[Ca^{2+}]$ [9, 10]. Recently, we demonstrated that the lack of SIK1 contributes to the vascular remodelling processes, e.g., dysregulated collagen synthesis and increased contractile phenotype of VSMCs, leading to increased vascular stiffness and consequently to higher blood pressure upon a chronic high-salt diet in mice [11].

The sympathetic nervous system plays a role in the regulation of cardiovascular function, and increased activation of the sympathetic nervous system has been claimed to be involved in the the pathophysiology of hypertension [12, 13], despite to date augmented sympathetic activity having been demonstrated only in a minority of hypertensive patients [14]. The increase in sympathetic activity has been demonstrated to be organ specific, in particular to the heart and kidneys, rather than being generalised, and is associated to increase mortality [14]. Evidence has also been obtained that sympathetic activation participates in the development of hypertension-related target organ damage, such as left ventricular diastolic dysfunction, left ventricular hypertrophy and arterial remodelling and hypertrophy [15]. Sympathetic cardiovascular influences may favour the hypertensive phenotype, by concurring with other hemo-dynamic and non-hemodynamic factors at the development of target organ damage [16, 17].

Dopamine β-hydroxylase (DβH) is the enzyme that catalyses the hydroxylation of dopamine (DA) to noradrenaline (NA) in the sympathetic nervous system. DβH inhibition causes sympathetic slowdown by reducing NA levels, and additionally leads to increased DA availability. The increase in DA levels can further promote renal vasodilation, natriuresis and diuresis [18, 19]. Etamicastat (development code BIA 5-453) is a peripherally selective and reversible inhibitor of DβH, currently under clinical development for the treatment of hypertension and heart failure [20–22]. Etamicastat displays a mixed (non-competitive) type DβH inhibition with respect to DA with a low nM Ki value. In contrast to what its found in the peripheral tissues, etamicastat does not affect DA or NA tissue levels in the brain [22]. Etamicastat, when administered to spontaneously hypertensive rats (SHR), produced a dose-dependent reduction in both systolic and diastolic blood pressures [23]. Additionally, chronic administration of etamicastat in drinking water, significantly reduced both blood pressure and urinary excretion of NA in SHR [20].

In the present study, we investigated the effect of SIK1 ablation on blood pressure after a 7-day high-salt diet in mice, focusing on the renal and sympathetic mechanisms of salt-induced hypertension.

Methods

Animal care and general procedures

The $sik1^{-/-}$ mice were purchased from Taconic Biosciences Inc. (Model [#]TF1350, Rensselaer, NY, USA) and previously described [4]. Colony was maintained on a heterozygous breeding scheme. Homozygous male SIK1 knockout ($sik1^{-/-}$) and wild-type ($sik1^{+/+}$) littermate mice were used in this study. Animals were housed in macrolon cages (Tecniplast, Varese, Italy) with free access to food ([#]2014 Teklad Global Rodent Diets®, Envigo, Barcelona, Spain) and tap water under controlled environmental conditions in a colony room (12 h light/dark cycle, room temperature: $22 \pm 2\,^{\circ}C$ and relative humidity: $50 \pm 20\%$) until the beginning of the experiments. Eight-week-old male $sik1^{-/-}$ and $sik1^{+/+}$ littermate control mice were challenged either a high-salt (8% NaCl, [#]D02011103) or control (0.3% NaCl, [#]D02112603) AIN-76A rodent diet (Research Diets Inc., New Brunswick, NJ, USA) for 7 days. Animal procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and the Portuguese law on animal welfare (Decreto-Lei 113/2013).

Blood pressure measurement

Implantable telemetry was used for blood pressure, heart rate and home-cage activity assessment. Mice were anaesthetised by intraperitoneal injection (10 ml/kg of body weight) of ketamine (150 mg/kg of body weight), medetomidine (1 mg/kg of body weight) and butorphanol (1 mg/kg of body weight) in the normal saline solution. Mice were instrumented with radio-telemeters ([#]TA11PA-C10, Data Sciences International, St Paul, MN, USA), as described elsewhere [11, 24]. Briefly, telemetry transmitter was inserted into the carotid artery after cranial permanent ligature and temporary caudal occlusion. Catheter tip was positioned and secured in the aortic arch. Post-operative care: wound closure was covered with 2% lidocaine cream and carprofen (5 mg/kg/day s.c.) was administered for 3 days, twice daily. Animals recovered individually before experiments. After recovery, telemetry probes were magnetically turned-on and blood pressure, heart rate and home-cage locomotor activity were monitored. Raw data were

recorded for 40 s every 10 min for 48 h using Dataquest A. R.T. Acquisition and Analysis system 4.0 (Data Sciences International).

Renal function

After telemetry recording, mice were individually placed in mouse metabolic cages (Tecniplast) for a 24-h urine collection. The volume of water intake and urinary excretion was noted. The urine samples were collected and stored at −80 °C until assayed.

Biochemical parameters

After completion of the protocol, mice were weighed and anaesthetised (60 mg/kg of body weight, i.p.) with sodium pentobarbital (Merck, Darmstadt, Germany) and killed by abdominal *vena cava* exsanguination. Blood samples (≈600 μl) were collected (Multivette® 600 Z, Sarstedt, Nümbrecht, Germany) and centrifuged (4 °C, 10,000 g, 5 min). Aliquoted serum samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until analyses. The heart, abdominal aorta and kidneys were exposed, rapidly excised and blotted dry; atria, ventricles and kidneys weight was recorded. Kidney mass weight-to-body weight ratio was used as an index of renal hypertrophy. All biochemical assays were performed by Cobas Mira Plus analyzer (ABX Diagnostics for Cobas Mira, Basel, Switzerland). Serum aldosterone levels were measured using a commercially available ACTIVE® Aldosterone ^{125}I radioimmunoassay (RIA) kit (Beckman Coulter, Inc., Brea, CA, USA, ref. DSL8600) according to the manufacturer's instruction and quantified on a gamma counter. Serum renin (Merck, Mouse Ren1/Renin-1 ELISA Kit, ref. RAB0565) and angiotensin II (Merck, Angiotensin II EIA Kit ref. RAB0010) levels were quantified by commercially available standard immunoassay kits following the manufacturer's instructions.

Assay of catecholamines

NA and DA levels were quantified in the kidney cortex, atria, ventricles and abdominal aorta. NA, DA, L-3,4-dihydroxyphenylalanine (L-DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC) levels were quantified in 24-h urine samples. All samples were analysed using a high-performance liquid chromatography system (Gilson Inc., Middleton, WI, USA) with electrochemical detection (HPLC-ED) as previously described [25].

Assay of DβH activity

DβH activity was determined in adrenal glands homogenates. In brief, adrenal glands were removed, placed in 200 μL of 50 mM Tris (Merck), pH 7.4 and stored at −80 °C until analysis. Determination of DβH activity was performed according to the method of Nagatsu and Udenfriend [26]. DβH activity was assessed by measuring the quantity of octopamine formed (expressed in ng/mg of protein/min) as described before [21].

mRNA expression

Kidney tissue samples were directly incubated with RNA-later (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA was extracted with RNeasy tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, with an additional step for genomic DNA removal. Quantification was performed on a 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) using RNA 6000 Nano LabChip Kits (Agilent Technologies). All samples had a RIN value ≥ 7.5 and concentration of 1.3–2.8 μg/μl. In total, 0.5 μg of RNA were reverse-transcribed using high capacity cDNA reserve transcription kit (Applied Biosystems Inc., Foster City, CA, USA), per the manufacturer's instructions. For gene expression assay, a Taq-Man array 96-well plate fast plate custom format 16 plus candidate endogenous control genes (PN4413262) was used. This array included three mouse endogenous genes (Gapdh, Hprt1 and Gusb) and 12 mouse inventoried genes (related to renal regulation of Na^+ balance). In total, 0.5 μg of cDNA per sample were amplified using the 2X TaqMan Fast Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems), per the manufacturer's instructions. qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). Raw data were analysed with DataAssist software v.3.01 (Applied Biosystems) using the Δ-ΔCt method (relative quantification). The relative amount of the mRNA of interest was normalized against to Hprt1 mRNAs using the comparative Ct method.

Treatment with etamicastat

Etamicastat was synthesized in the Laboratory of Chemistry of BIAL-Portela & Cª, S.A. [Coronado (S. Romão e S. Mamede)] with a purity grade > 96% [27]. Mice were administered 50 mg/kg/day etamicastat in drinking water. Etamicastat treatment started 7 days before high-salt intake and lasted until the end of the experiment. The daily dose of etamicastat attained for the entire experimental period was 55.6 ± 1.6 mg/kg of body weight.

Statistical analysis

The data are presented as the mean ± SEM. Data analyses were performed using Prism 6 (GraphPad Software, San Diego, CA, USA). The data were analysed by one-way

analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test or unpaired t test, as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

### Loss of SIK1 increases blood pressure upon an acute high-salt intake in mice

Blood pressure and heart rate recordings were performed on telemetered $sik1^{-/-}$ and $sik1^{+/+}$ littermate mice challenged to either a high-salt or control diet for 7 days. No differences in blood pressure were observed between $sik1^{-/-}$ and $sik1^{+/+}$ mice on a control diet (Fig. 1 and Supplementary Fig. S1). However, upon a 7-day high-salt intake, systolic blood pressure was significantly elevated in $sik1^{-/-}$ mice (137 ± 7 mm Hg) as compared with $sik1^{+/+}$ mice (121 ± 2 mm Hg, $P = 0.0054$) and to $sik1^{-/-}$ mice on a control diet (125 ± 7 mm Hg, $P = 0.0218$). Likewise, mean arterial pressure was significantly elevated in $sik1^{-/-}$ mice (122 ± 9

mm Hg) compared with $sik1^{+/+}$ mice (106 ± 3 mm Hg, $P = 0.0265$) on a high-salt diet, whereas diastolic blood pressure was similar in both groups (Fig. 1 and Supplementary Fig. S1). No differences in heart rate and home-cage activity were observed between groups (Fig. 1 and Supplementary Fig. S1).

Renal function and the renin–angiotensin system in the $sik1^{-/-}$ mice

Body weight, kidney hypertrophy and urinalysis in $sik1^{-/-}$ and $sik1^{+/+}$ mice on a control and high-salt diet are summarized in Table 1. SIK1 ablation had no effect on body weight. Conversely, on a high-salt diet, $sik1^{+/+}$ mice were slightly, but significantly, heavier than their counterparts on a control diet. Kidney weight-to-body weight ratio in $sik1^{-/-}$ mice was significantly higher, under both diet protocols as well as in wild-type mice fed a high-salt diet. Water intake and urinary volume output were similar between both genotypes, under both diet protocols. Upon high-salt intake, the water consumption increased by 2.8- and 2.1-fold in both genotypes, $sik1^{+/+}$ and $sik1^{-/-}$ mice, respectively, and

Fig. 1 Blood pressure, heart rate and home-cage activity in telemetered salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) and wild-type ($sik1^{+/+}$) littermate mice challenged either a high-salt (8% NaCl) or control (0.3% NaCl) diet for 7 days. (**a**) Systolic blood pressure (SBP), (**b**) diastolic blood pressure (DBP), (**c**) mean arterial pressure (MAP), (**d**) heart rate (HR) and (**e**) home-cage activity. Mean ± SEM; $n = 6$–9 per group; values were determined to be significantly different from $sik1^{+/+}$ mice (*$P < 0.05$) or control diet-fed counterparts ($^{\#}P < 0.05$) group by one-way analysis of variance followed by Fisher's least significant difference (LSD) test
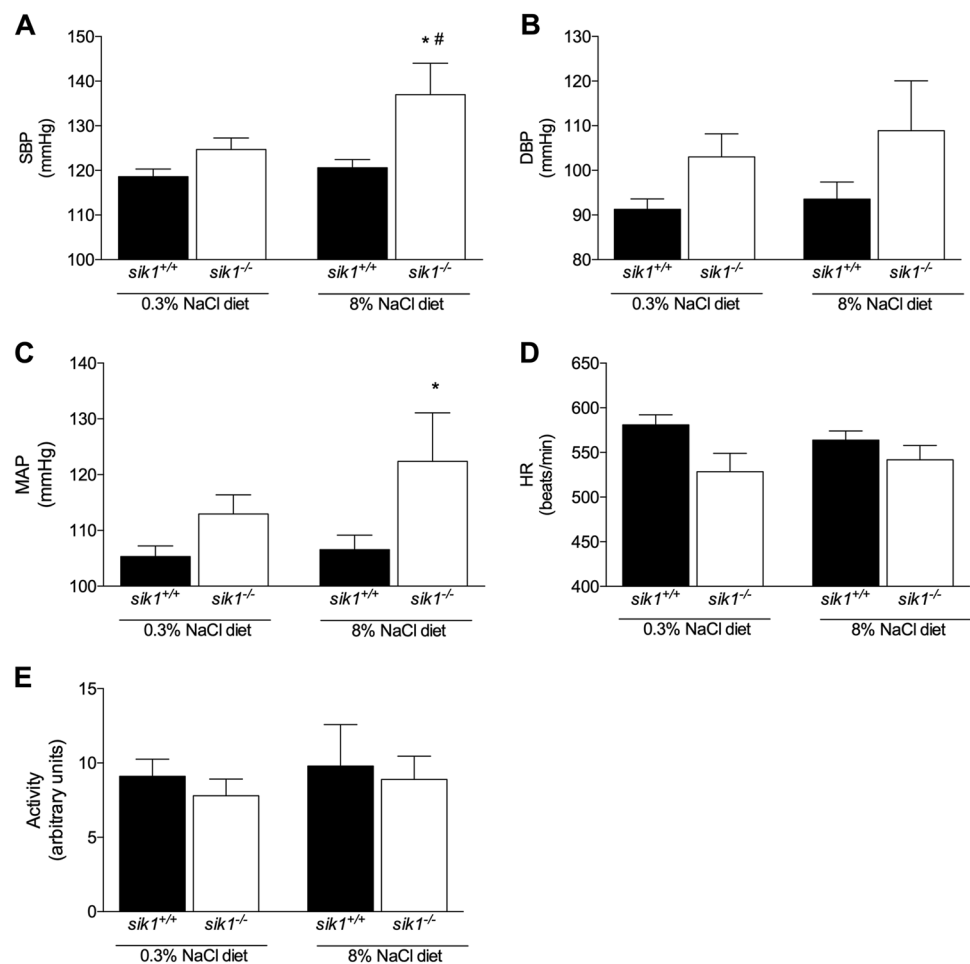
Table 1 Renal function after 7 days of 0.3 or 8% NaCl intake in $sik1^{+/+}$ and $sik1^{-/-}$ mice

| | 0.3% NaCl diet | | 8% NaCl diet | |
	$sik1^{+/+}$	$sik1^{-/-}$	$sik1^{+/+}$	$sik1^{-/-}$
Body weight (g)	25.7 ± 0.6	25.8 ± 1.1	29.2 ± 0.9[#]	28.2 ± 0.5
Kidney-to-body weight ratio (mg/g)	11.3 ± 0.2	13.0 ± 0.2*	12.6 ± 0.2[#]	13.9 ± 0.2*
Water intake (ml/24 h)	2.5 ± 0.2	3.3 ± 0.5	6.9 ± 0.6[#]	6.8 ± 0.7[#]
Urinary output (ml/24 h)	1.6 ± 0.2	1.0 ± 0.2	3.5 ± 0.7[#]	4.3 ± 0.6[#]
Urine creatinine (mg/24 h)	0.62 ± 0.03	0.48 ± 0.04	0.62 ± 0.04	0.57 ± 0.05
Urine sodium/creatinine ratio (μmol/mg)	119 ± 8	107 ± 11	2075 ± 450[#]	3041 ± 251* [#]
Urine potassium/creatinine ratio (μmol/mg)	220 ± 10	183 ± 13	251 ± 27	341 ± 15* [#]
Urine chloride/creatinine ratio (μmol/mg)	403 ± 36	405 ± 43	1719 ± 340[#]	2268 ± 157[#]

Mean ± SEM, n = 5–6 per group. *P < 0.05 as compared with $sik1^{+/+}$ mice; [#]P < 0.05 as compared with 0.3% NaCl feeding counterparts

Table 2 Serum renin, angiotensin II and aldosterone levels after 1 week of 0.3 or 8% NaCl intake in $sik1^{+/+}$ and $sik1^{-/-}$ mice

| | 0.3% NaCl diet | | 8% NaCl diet | |
	$sik1^{+/+}$	$sik1^{-/-}$	$sik1^{+/+}$	$sik1^{-/-}$
Renin	465 ± 22	463 ± 18	462 ± 33	463 ± 32
Angiotensin II	33 ± 7	46 ± 10	44 ± 7	39 ± 9
Aldosterone	267 ± 67	197 ± 30	83 ± 9[#]	78 ± 6[#]

All = pg/ml. Mean ± SEM, n = 3–6 per group. [#]P < 0.05 as compared with 0.3% NaCl feeding counterparts

this was accompanied by an increase in urinary volume output. Under control diet feeding, urinary creatinine levels were not different between $sik1^{+/+}$ (0.62 ± 0.03 μmol/mg) and $sik1^{-/-}$ mice (0.48 ± 0.04 μmol/mg), moreover high-salt intake had no effect on urinary creatinine (0.62 ± 0.04 and 0.57 ± 0.05 μmol/mg, respectively). Likewise, no differences between genotypes were observed in the daily urinary excretion of sodium, potassium or chloride on a control diet. A high-salt intake significantly increased urinary sodium and chloride output in both genotypes. Conversely, the urinary potassium levels were 1.9-fold higher exclusively in $sik1^{-/-}$ mice after a high-salt diet. Likewise, sodium and potassium excretion were significantly increased in $sik1^{-/-}$ animals, but not in wild-type control mice, under a high-salt intake.

Serum renin, angiotensin II and aldosterone levels were similar between $sik1^{-/-}$ and wild-type mice, under both salt regimens. Serum aldosterone levels significantly decreased in the high-salt diet protocol, in a similar extent, in both genotypes (Table 2).

To further assess the causes of the increased blood pressure in $sik1^{-/-}$ mice upon a high-salt intake, the expression of genes related to renal regulation of sodium, was evaluated in kidney tissue samples. There was no difference in gene expression levels between $sik1^{-/-}$ and $sik1^{+/+}$ mice fed a control or high-salt diet (Supplementary Table S1).

Sympathetic activity upon acute high-salt intake in the $sik1^{-/-}$ mice

Catecholamine levels were assessed in urine and in several peripheral organs. Regarding tissue levels of catecholamines, no significant changes were detected in the kidney, atria, ventricles and aorta of $sik1^{-/-}$ and $sik1^{+/+}$ mice fed either control or high-salt diet for 7 days (Table 3). Twenty-four-hour urine was collected in metabolic cages, after control or high-salt diet feeding in $sik1^{+/+}$ and $sik1^{-/-}$ mice. As shown in Fig. 2a, NA excretion was 58% decreased in urine samples from the $sik1^{-/-}$ mice on a control diet, but increased 2.9-fold after high-salt intake. As depicted in Fig. 2, an increase of DA metabolites, DOPAC and its precursor L-DOPA, were also reported during high-salt intake, but no significant differences were detected in DA levels.

DβH activity was evaluated in adrenal gland homogenates. Under a control diet, DβH activity was similar in both genotypes. Upon challenge with a high-salt diet, DβH activity increased in both $sik1^{-/-}$ and wild-type mice, but to a greater extent in the $sik1^{-/-}$ mice (Fig. 3).

DβH activity and catecholamine levels were measured in knockout mice fed a high-salt diet after pre-treatment with etamicastat, a reversible inhibitor of peripheral DβH. In the $sik1^{-/-}$ mice on a high-salt diet regimen, etamicastat led to a 59% decreased in the adrenal DβH activity (Fig. 4). Regarding catecholamine levels in urine, kidney, ventricles, atria and aorta, etamicastat reduced NA in urine and in all tissues analysed in $sik1^{-/-}$ mice after high-salt intake (Fig. 5 and Table 4). Etamicastat had no effect on urinary L-DOPA levels (Fig. 5). On the other hand, DA and DOPAC levels were increased in both urine and tissues (Fig. 5 and Table 4).

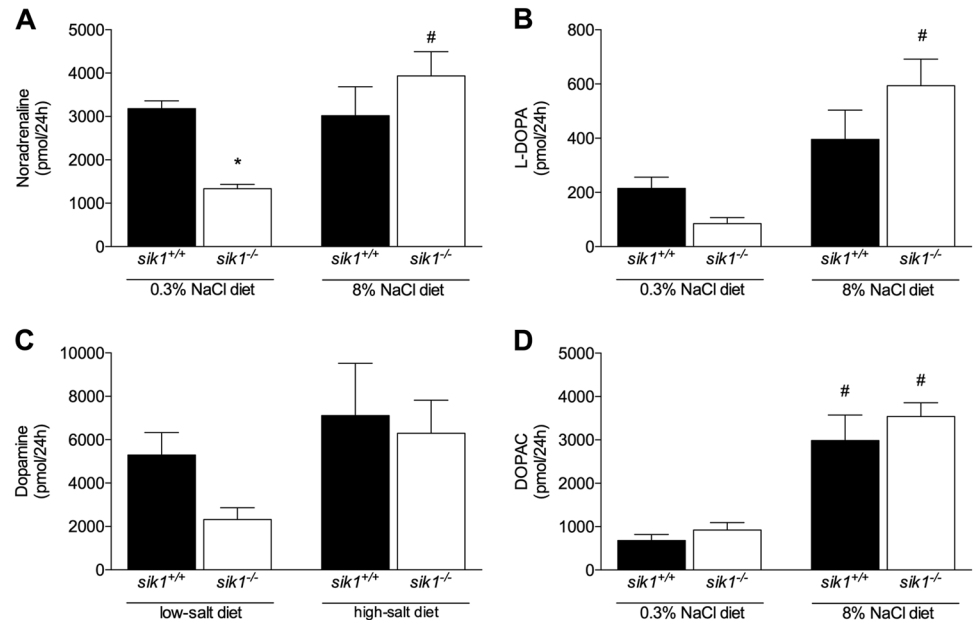## Etamicastat and blood pressure in the $sik1^{-/-}$ mice

Blood pressure and heart rate recordings were assessed on telemetry-implanted $sik1^{-/-}$ mice after 14 days of treatment

Table 3 Tissue catecholamine levels after 1 week of 0.3 or 8% NaCl intake in $sik1^{+/+}$ and $sik1^{-/-}$ mice

	0.3% NaCl diet		8% NaCl diet	
	$sik1^{+/+}$	$sik1^{-/-}$	$sik1^{+/+}$	$sik1^{-/-}$
Kidney				
Noradrenaline	2291 ± 195	2653 ± 222	2603 ± 137	2878 ± 232
Dopamine	423 ± 43	381 ± 42	331 ± 11	351 ± 58
Ventricles				
Noradrenaline	2962 ± 183	2559 ± 161	2765 ± 163	2629 ± 240
Dopamine	217 ± 18	169 ± 8	175 ± 7	153 ± 15
Atria				
Noradrenaline	6590 ± 673	7017 ± 1026	7047 ± 625	7474 ± 728
Dopamine	833 ± 121	754 ± 73	746 ± 38	836 ± 102
Aorta				
Noradrenaline	6799 ± 992	8128 ± 971	8215 ± 490	9337 ± 1933
Dopamine	681 ± 62	661 ± 55	799 ± 73	727 ± 112

All = pmol/g tissue. Mean ± SEM, $n = 5$ per group

Fig. 2 Urinary L-DOPA, catecholamines and metabolites in salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) and wild-type ($sik1^{+/+}$) littermate mice challenged either a high-salt (8% NaCl) or control (0.3% NaCl) diet. **a** Noradrenaline, (**b**) L-DOPA, (**c**) dopamine and (**d**) 3,4-dihydroxyphenylacetic acid (DOPAC). Mean ± SEM; $n = 6$ per group; values were determined to be significantly different from $sik1^{+/+}$ mice (*$P < 0.05$) or from control diet-fed counterparts ($^{\#}P < 0.05$) group by one-way analysis of variance followed by Fisher's least significant difference (LSD) test


with etamicastat, in which in the last 7 days, animals were fed a high-salt diet. As depicted in Fig. 6, etamicastat treatment prevented the increase in systolic blood pressure caused by a 7-day high-salt diet feeding in $sik1^{-/-}$ mice. A small, but significant, decrease in heart rate was also observed after etamicastat treatment (Fig. 6 and Supplementary Fig. S2).

Discussion

Here, we proposed to study the role of SIK1 on blood pressure rise after a 7-day period of high-salt intake, namely by evaluating the role of renal and sympathetic mechanisms. Our results suggest that SIK1 has a direct effect on blood pressure regulation via the sympathetic nervous system. These results confirm and extend our previous observations that vascular SIK1 activation might represent a mechanism involved in the prevention of high blood pressure and highlights the relevance of SIK1 in blood pressure regulation in vivo [6, 11].

SIK1 modulates NKA activity and contributes to the reabsorption of sodium in the kidney proximal tubules. This is in agreement with the increased natriuresis observed after 7 days on high-salt diet, where $sik1^{-/-}$ mice excreted 1.5-fold more sodium than $sik1^{+/+}$ mice on the same diet regimen, as creatinine excretion rates were similar. This difference in renal electrolyte handling should have led to a lower blood pressure in $sik1^{-/-}$ mice as compared with $sik1^{+/+}$ mice feed a high-salt diet. Nevertheless, the contribution of
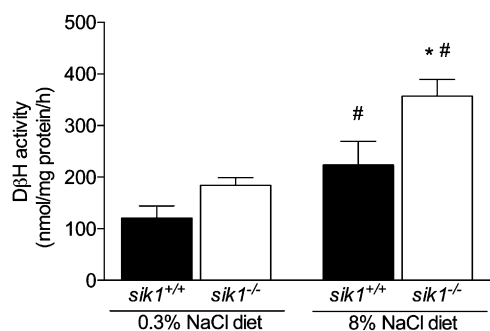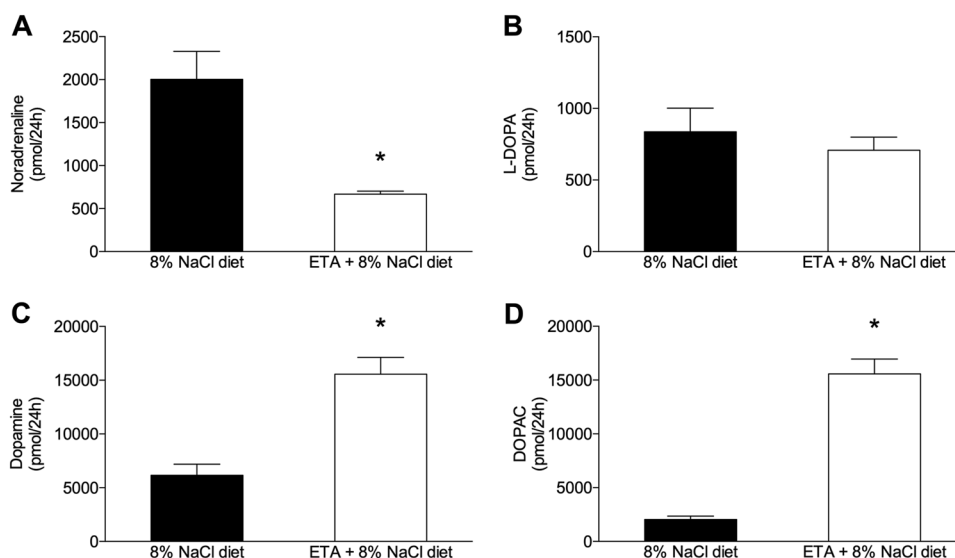
Fig. 3 Dopamine β-hydroxylase (DβH) activity in adrenal glands of salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) and wild-type ($sik1^{+/+}$) littermate mice challenged either a high-salt (8% NaCl) or control (0.3% NaCl) diet. Mean ± SEM; $n = 5$ per group; values were determined to be significantly different from $sik1^{+/+}$ mice (*$P < 0.05$) or from control diet-fed counterparts ($^{#}P < 0.05$) group by one-way analysis of variance followed by Fisher's least significant difference (LSD) test
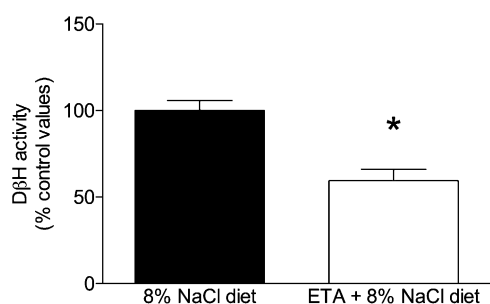
Fig. 4 Effect of etamicastat (ETA) treatment on dopamine β-hydroxylase (DβH) activity in adrenal glands of salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) mice challenged with a high-salt (8% NaCl) diet. Mean ± SEM; $n = 7$ to 8 per group; values were determined to be significantly different from high-salt diet group (*$P < 0.05$) by unpaired t test

SIK1 in controlling the vascular tone [6, 8, 11] seems to surpass the contribution of renal SIK1 which could also be compensated by other SIK isoforms mechanisms, leading to a higher blood pressure in $sik1^{-/-}$ than in $sik1^{+/+}$ mice, under a high-salt diet [11].

In an attempt to uncover the mechanisms that may be responsible for the increase in blood pressure upon high sodium diet in the $sik1^{-/-}$ mice, the renin–angiotensin system was evaluated as a potential trigger for the rise in blood pressure. On a high-salt regimen, serum aldosterone levels were significantly lower in both $sik1^{+/+}$ and $sik1^{-/-}$ mice compared with mice on a control diet, indicating a negative feedback loop of the renin–angiotensin system. Despite the significant increase in sodium excretion and a

Table 4 Tissue catecholamines levels after one week of 50 mg/kg/day etamicastat (ETA) treatment in $sik1^{-/-}$ mice on an 8% NaCl intake

	8% NaCl diet	ETA + 8% NaCl diet
Kidney		
Noradrenaline	2437 ± 169	1136 ± 98*
Dopamine	408 ± 29	1169 ± 72*
Ventricles		
Noradrenaline	3723 ± 224	1736 ± 224*
Dopamine	298 ± 30	1672 ± 178*
Atria		
Noradrenaline	8913 ± 987	2484 ± 187*
Dopamine	898 ± 126	2500 ± 98*
Aorta		
Noradrenaline	7315 ± 1431	3209 ± 444*
Dopamine	968 ± 186	5707 ± 340*

All = pmol/g tissue. Mean ± SEM, $n = 7$ per group. *$P < 0.05$ as compared with 8% NaCl diet group

Fig. 5 Effect of etamicastat (ETA) treatment on urinary L-DOPA, catecholamines and metabolites in salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) mice challenged with a high-salt (8% NaCl) diet. **a** noradrenaline, (**b**) L-DOPA, (**c**) dopamine and (**d**) 3,4-dihydroxyphenylacetic acid (DOPAC). Mean ± SEM; $n = 7$–8 per group; values were determined to be significantly different from 8% NaCl diet group (*$P < 0.05$) by unpaired t test

Fig. 6 Effect of 50 mg/kg/day etamicastat (ETA) treatment on blood pressure, heart rate and home-cage activity in telemetered salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) mice challenged with a high-salt (8% NaCl) diet. ETA treatment for 14 days, in which in the last 7 days, animals were fed a high-salt diet. **a** Systolic blood pressure (SBP), (**b**) diastolic blood pressure (DBP), (**c**) mean arterial pressure (MAP), (**d**) heart rate (HR) and (**e**) home-cage activity. Mean ± SEM; $n = 5$ to 6 per group; values were determined to be significantly different from high-salt diet group (*$P < 0.05$) by unpaired t-test
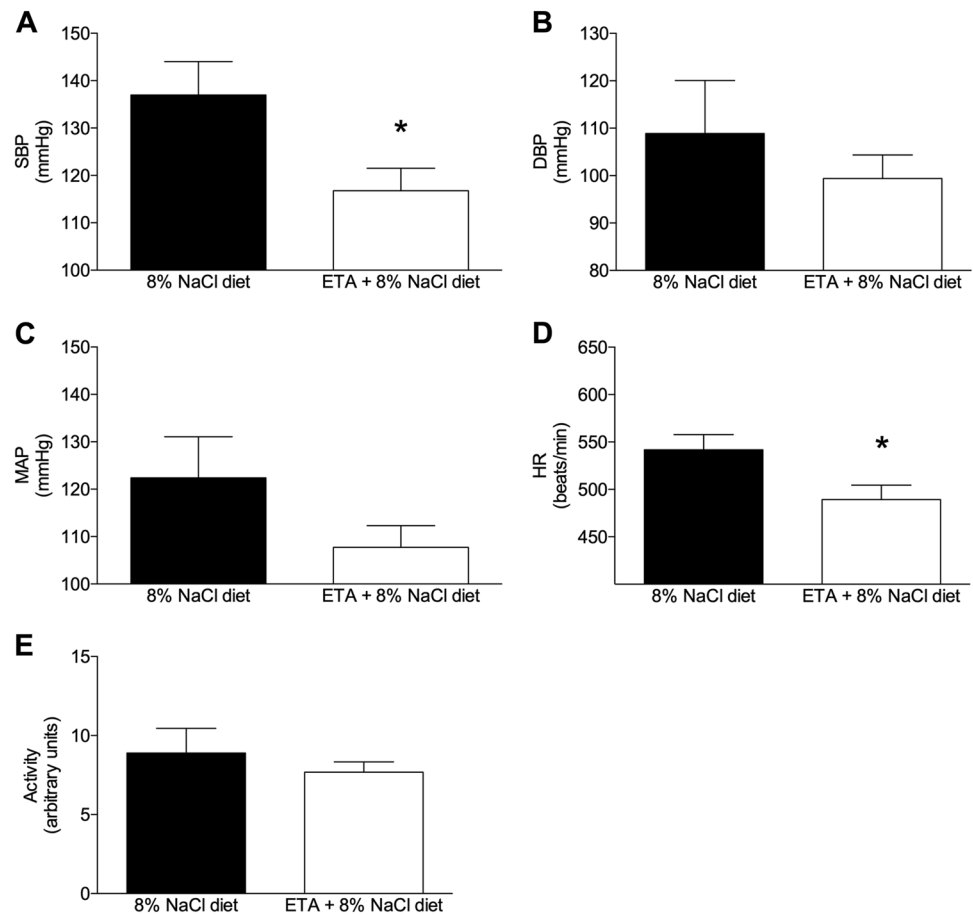
decrease in serum aldosterone levels, no decrease in circulating renin was observed. The effect of high-salt diet on renin activity is unknown as only circulating renin mass was addressed. A possible explanation for this conundrum is that blood samples were obtained from pentobarbital-anaesthetised mice. Meneton et al. have shown that plasma renin concentration is increased by pentobarbital anaesthesia [28]. Nonetheless, no differences between genotype in serum renin levels were found on both diet protocols, suggesting that the loss of SIK1, under a high-salt intake, leads to an altered electrolyte excretion and to heavier kidneys without the involvement of the renin–angiotensin system pathway. This is in line with the finding that the C57BL/6 mouse strain appear to be resistant to hypertension-induced renal injury [29] and renal injury caused by angiotensin II infusion, protein load or renal ablation [30–32]. Moreover, abnormalities in the renin–angiotensin system do not seem to be implicated in the salt sensitivity of C57BL/6 mice [33].

To evaluate whether the sympathetic nervous system contributes to the elevation of blood pressure observed in the $sik1^{-/-}$ mice during a high-salt diet, catecholamine levels in urine and in several peripheral tissues were measured. Urinary NA, DA and L-DOPA levels were reduced

more than 50% in the $sik1^{-/-}$ mice compared with the wild-type counterparts. SIK1 is a well-studied cell sodium-sensing mechanism that controls intracellular sodium concentration by regulating NKA activity. Therefore, a possible explanation for the reduced urinary L-DOPA levels in the SIK1$^{-/-}$ mice could be a reduction in the intracellular transport of L-DOPA into kidney cells as a consequence of a decreased NKA activity. The impaired transport of L-DOPA would also account for the reduced levels of DA, since uptake of L-DOPA is the rate-limiting step for DA synthesis in the kidney. However, basal NKA activity, at normal intracellular sodium, remains unaffected under conditions when SIK1 activity is suppressed, which indicates that in the absence of SIK1, reduced L-DOPA transport does not account for the changes in L-DOPA and DA [6]. The reduction in L-DOPA levels due to a reduction in catecholamine synthesis can better account for this reduction, as well as in overall sympathetic NA.

Plasmatic L-DOPA is derived substantially, albeit not exclusively, from catecholamine biosynthesis from sympathetic nerves endings [34]. Tyrosine hydroxylase (TH) is the enzyme responsible for the conversion of tyrosine to L-DOPA and is considered the rate-limiting step for catecholamine synthesis. TH is highly regulated at the post-

translational level by phosphorylation at several serine residues [35]. Phosphorylation of TH is associated with an increased enzymatic activity and plays a key role in the regulation of the sympathetic system. SIK1 belongs to the family of AMPK and has been shown to phosphorylate TH, thereby potentially regulating the activity of this enzyme [36]. Absence of SIK1 could result in downregulation of TH phosphorylation leading to a reduction in L-DOPA synthesis and release from the sympathetic terminals, that could account for the lower levels of the catecholamine precursor in urine. Reduction of catecholamine synthesis in sympathetic terminals would also account for the fact that NA levels in urine was reduced in $sik1^{-/-}$ as compared with $sik1^{+/+}$ mice. On the other hand, in this study no significant changes in NA levels in cardiovascular and kidney tissue or in plasma NA levels were observed as previously described [11].

NA levels in sympathetic terminals are constantly fluctuating due to the highly dynamic regulation of its synthesis, release, re-uptake and metabolism [37]. Moreover, the majority of NA in sympathetic terminals is stored in vesicles, and only a small percentage (about 10%) being syphoned to the circulation. It was therefore not surprising that, in the $sik1^{-/-}$ mice, the tissue levels of NA were unaltered, which is in line what we previously observed in the plasma [11]. On contrary, levels in the urine provide a good indication of overall changes that occur in sympathetic drive by taking into account fluctuations that occur over a 24-h period.

Interestingly, DβH activity in adrenals was found not to be altered, and even with a small tendency to be increased. Unlike TH, DβH is not subjected to tight regulation but nevertheless plays a crucial role in NA synthesis, as it is responsible for the conversion of DA to NA [38].

In control diet groups, $sik1^{-/-}$ mice had lower urinary NA than wild-type mice, whereas there was no difference in systolic blood pressure between these two groups. In high-salt diet groups, on the other hand, $sik1^{-/-}$ mice displayed a higher systolic blood pressure than $sik1^{+/+}$ mice while their urinary NA is similar. The increase in blood pressure observed in the $sik1^{-/-}$ mice after high-salt diet, is likely to be caused by a burst of sympathetic activity as revealed by the increase in urinary excretion of NA and by the increase in adrenal DβH activity. Upon high-salt intake, there was a significant increase in L-DOPA and NA levels in the $sik1^{-/-}$ mice, but not in wild-type littermates. As stated above, fluctuations in L-DOPA levels are likely to be due to an increase in synthesis by changes in the enzyme responsible for catecholamine synthesis, TH. In fact, increased sympathetic TH activity and increased NA levels have been demonstrated in the kidney of a rodent model of hypertension [39]. The inability to inhibit noradrenergic activity may lead to prolonged peripheral vasoconstriction,

ultimately leading to hypertension. Moreover, a significant increase in DβH activity was observed in both, $sik1^{-/-}$ and $sik1^{+/+}$ mice, but the overall activity was higher in the $sik1^{-/-}$ mice. Therefore, in the $sik1^{-/-}$ mice the high-salt intake triggers a surge in sympathetic activity, leading to an increase in NA synthesis and release. In light of this, it could be argued that SIK1 has evolved to act as the buffering system for sympathetic nervous system activity. The observed overdrive of the sympathetic nervous system, namely the noradrenergic and adrenergic tone in the SIK1-null mice may partially explain the increase in blood pressure triggered by a high-salt intake, which is also consistent with the over-activation of DβH. These results reveal a determinant role of SIK1 in the sympathetic nervous system upon an acute high-salt intake.

Etamicastat is a potent and reversible inhibitor of DβH that prevents the conversion of DA to NA in peripheral sympathetically innervated tissues, thereby decreasing the sympathetic nervous system drive [17]. In contrast to what is found in the peripheral tissues, etamicastat does not affect DA and NA levels in the brain [22]. Etamicastat was tested in animal models predictive of efficacy of drugs in cardiovascular disorders. In SHR, etamicastat reduced systolic and diastolic blood pressure alone or in combination with anti-hypertensive drugs [20, 23]. In this study, administration of etamicastat prevented the rise in blood pressure in the $sik1^{-/-}$ mice fed with a high-salt intake. As expected, treatment with etamicastat produced a significant decrease in NA levels and a significant increase in DA levels in cardiovascular tissues, which is associated with the inhibition of DβH activity. In the kidney, treatment with etamicastat also produced the same effect as seen in the cardiovascular tissue, a decrease in NA levels and an increase in DA levels. This effect is most likely due to the inhibition of DβH in sympathetic nerve terminals since kidney cells are not endowed with DβH and synthesize dopamine from circulatory L-DOPA. Overall, the reduction in sympathetic activation was accompanied by a significant decrease in NA levels and increase in DA levels in the urine. The latter could be attributed in part to the inhibition of DβH, but since DA found in urine derives mostly from newly synthesized DA in the kidney, it is more likely that the increase in DA urinary levels is due to increased renal synthesis [40]. The increase in DA and the decrease in NA leads to an increase in the DA-to-NA ratio, that can be taken as a natriuretic index. In a situation when sodium excretion needs to be enhanced, the DA-to-NA ratio should be increased, as the two hormones have opposite actions on sodium reabsorption and vascular resistance [41]. The inability of the kidney to respond to the increased dopaminergic system activity, through increased dopamine synthesis, could further underscore the idea that an increased activity of the noradrenergic system may be

responsible for the imbalance in sodium, ultimately leading to manifest hypertension [42, 43].

Taken together, the data presented here support the view that SIK1, an intracellular sodium-sensing mechanism, by regulating the activity of the sympathetic nervous system affects blood pressure. Efforts aimed at decreasing sympathetic drive by targeting SIK1, could prove to be an interesting strategy in the management, treatment/prevention of sodium sensitive hypertension that results from an imbalance of renal sodium concentration.

Compliance with ethical standards

Conflict of interest NMP, BI, EM, FLC and PS-d-S are or were employees of BIAL-Portela & Cª, S.A. at the time of the study.

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