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MESTRADO INTEGRADO EM MEDICINA

2018/2019

Emanuel Fernando Pinto Guedes Lopes Matias

Effects of salt-inducible kinase 1 (SIK1) ablation on intestinal ion transport modulation

março, 2019

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Effects of salt-inducible kinase 1 (SIK1) ablation on intestinal ion transport modulation

Mestrado Integrado em Medicina

Área: Ciências médicas e da saúde

Tipologia: Dissertação

Trabalho efetuado sob a Orientação de:

Professor Doutor Patrício Manuel Vieira Araújo Soares da Silva

Trabalho organizado de acordo com as normas da revista:

Hypertension Research

março, 2019

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DESIGNAÇÃO DA ÁREA DO PROJECTO

Ciências médicas e da saúde

TÍTULO DA DISSERTAÇÃO

Effects of salt-inducible kinase 1 (SIK1) ablation on intestinal ion transport modulation

ORIENTADOR

Professor Doutor Patrício Manuel Vieira Araújo Soares da Silva

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A toda a minha família, em especial:

pais, Maria e Fernando;

avós, Lucília e Manuel;

madrinha, Porcina;

tios, Isilda e Miguel.

Agradecimentos

Melhor caminho é aquele que dura e dista sem medir-se. Recostada por fim a capa sobre o presente trabalho, sinto ter em mãos – agora encadernados – os longos passos de uma das melhores decisões por mim já tomadas. No encalço agradecido da caminhada, outros pares de pés desenham-se adiante – a todos eles guardarei um sentido obrigado.

Em primeiro lugar, agradeço ao Professor Patrício Soares da Silva. Foi no seu carácter de pessoa e profissional de conhecimento, rigor e entrega que encontrei a determinação e certeza impulsoras para decidir-me por este desafio. Todo este percurso foi apenas possível graças ao seu apoio constante e atento. A ele o meu profundo obrigado pela oportunidade e pela confiança dadas.

Em segundo lugar, agradeço à Eng.ª Paula Serrão. Foi na sua experiência, disponibilidade e acolhimento que busquei a tranquilidade e segurança necessárias ao longo do trajeto. A ela o meu sincero obrigado pelo ombro amigo, firme e paciente.

Em terceiro lugar, agradeço a todos os elementos da Unidade de Farmacologia e Terapêutica, em especial aos que mais diretamente foram auxílio, ânimo e simpatia – Eng.ª Joana Afonso, Dr.ª Dina Cosme, Dr.ª Tatiana António, Dr.ª Catarina Carneiro, Dr.ª Ana Oliveira, Dr.ª Mafalda Santiago, D.ª Pilar Moreira e D.ª Conceição Martins.

Por último, porém sobretudo, agradeço aos meus pais. Desde sempre são fortaleza e arcaboiço robusto. Do sonho à montanha, tudo lhes devo.

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.

Ricardo Reis, *Odes*

Resumo

A hipertensão essencial é uma condição multifatorial e poligénica fortemente associada ao consumo excessivo de sal e à desregulação da absorção de sódio. A cínase 1 induzida pelo sal (*salt-inducible kinase 1* – SIK1), uma cínase da serina/treonina, é parte de uma rede intracelular de sinalização ao sódio que controla o transporte ativo deste ião através da regulação da atividade da bomba sódio-potássio (Na^+-K^+-ATPase), tendo sido identificada como um fator determinante na regulação da tensão arterial. Recentemente, foi demonstrado que a perda da SIK1 despoleta uma subida da tensão arterial em ratos submetidos a dieta hipersalina. No presente trabalho, o papel da SIK1 no desenvolvimento da hipertensão sensível ao sal foi adicionalmente explorado. Num conjunto específico de experiências, foi dado um primeiro passo na investigação da influência da SIK1 sobre a absorção de sódio no trato gastrointestinal, com foco na caracterização de mecanismos de transporte iónico intestinal potencialmente regulados por esta cínase, tais como o acoplamento funcional entre a bomba sódio-potássio e as correntes de potássio basolaterais (*pump-leak coupling*). Foram registadas as alterações no potencial de membrana após a exposição de células a fármacos conhecidos por abrir ou fechar os canais de potássio sensíveis ao ATP, tendo também sido avaliados os efeitos do tratamento com dexametasona sobre a condutância basolateral de potássio e sobre a expressão e atividade da bomba sódio-potássio. Os nossos resultados demonstram que a dexametasona causa uma modulação dependente da dose nas correntes de potássio basolaterais, não associada à sobre-expressão da bomba sódio-potássio e provavelmente secundária ao aumento da atividade desta enzima, em consequência do seu acoplamento funcional aos canais de potássio sensíveis ao ATP. Além disso, o nosso estudo descreve pela primeira vez uma aumento da expressão da bomba sódio-potássio em células epiteliais intestinais tratadas com concentrações superiores de dexametasona.

Abstract

Essential hypertension is a multifactorial and polygenetic condition strongly associated to high salt intake and sodium absorption dysregulation. Salt-inducible kinase 1 (SIK1), a sucrose nonfermenting-1-related serine/threonine kinase, is part of a cell sodium-sensing network that controls active sodium transport by regulating Na^+-K^+-ATPase activity and has been established as a key determinant in blood pressure regulation. Recently, loss of SIK1 has been shown to trigger a blood pressure rise in mice under chronic high salt intake. In the present work, we further explored the role of salt-inducible kinase 1 in sodium-sensible hypertension development. In a particular set of experiments, we took a first step in investigating the influence of SIK1 over transcellular sodium transport in the gastrointestinal tract, focusing on the characterization of intestinal ion transport mechanisms that are potentially under the regulation of SIK1 such as the pump-leak coupling between Na^+-K^+-ATPase and basolateral K^+ currents. Changes in membrane potential were examined after exposure of cells to drugs known to block or open ATP-sensitive K^+ channels and the effects of dexamethasone treatment on basolateral K^+ conductance and Na^+-K^+-ATPase protein expression and activity were assessed. Our results demonstrate that dexamethasone causes a concentration-dependent modulation of basolateral K^+ currents that is independent of Na^+-K^+-ATPase protein overexpression and most likely secondary to increased NKA activity, as a result of the functional coupling between Na^+-K^+-ATPase and K_{ATP} channels. Furthermore, we also report for the first time that dexamethasone induces Na^+-K^+-ATPase upregulation in intestinal epithelial cells at higher treatment concentrations.

Keywords

gastrointestinal tract, hypertension, pump-leak coupling, SIK1, transcellular sodium transport

General Introduction

Animal cells are highly permeable to water[1]. Any imbalance in intracellular or extracellular osmolarity is accompanied by a respective water movement across cell membrane and subsequent alterations in cell volume[2]. A number of volume regulatory mechanisms to avoid excessive variations in cell volume have been described, in particular ion transport across the cell membrane[2]. The ability to maintain a tightly regulated cell volume is dependent of the adequate ionic composition of the intracellular milieu in response to variations in the composition of the extracellular compartment[3]. Sodium is the most important determinant of water content in the body compartments, dragging water across membranes by osmolar differences[3]. In fact, sodium misdistribution can elicit abnormal water accumulation, leading to intra- and extracellular edema, hypervolemia and increased blood pressure, as occurs in patients with sodium-sensitive hypertension[4]. In order to control the water-attracting power of sodium and maintain homeostasis, cells compensate by actively pumping this ion towards the extracellular milieu[5], with water following isosmotically[6]. The major determinant of intracellular sodium concentration and the primary cell volume regulator is the plasma membrane sodium-and potassium-activated adenosine triphosphatase (Na^+-K^+-ATPase)[5].

The Na^+-K^+-ATPase (NKA) is a transmembrane heterodimer protein consisting of a catalytic α-subunit and a highly glycosylated β-subunit required for its integration into the plasma membrane and enzyme activity[7]. By using the energy-rich phosphate bound of a single ATP molecule, three sodium ions (Na^+) are actively translocated outward against two potassium ions (K^+) inward[8], thus establishing an electrochemical gradient across the plasma membrane[5, 7], which is necessary for cardiac and vascular contractility, neural transmission and transepithelial transport of solutes[7]. Therefore, Na^+-K^+-ATPase dictates active sodium transport and related vital functions[6], and is target of numerous regulatory mechanisms in response to changing cell requirements[5]. In transporting epithelia, phosphorylation of the Na^+-K^+-ATPase α-subunit has been associated with a reduction in active cell sodium transport, whereas dephosphorylation has been suggested to increase NKA catalytic activity in response to physiological or pharmacological stimuli[3, 9-11]. Many described effectors are known to physically interact with sodium pump subunits after sensing small increases in intracellular sodium[6], regulating its activity, trafficking

and signaling (Na^+-K^+-ATPase-regulatory complex)[12-18] – one recently identified molecule of this system is salt-inducible kinase 1 (SIK1)[6].

Salt-inducible kinase 1 (SIK1) was first identified in the adrenal cortex of rats receiving a sodium-enriched diet and interpreted as an important component in the adrenocortical response to high plasma Na^+, K^+, ACTH or stress[19]. This molecule was later revealed to be part of a cell sodium-sensing network and described as a sucrose nonfermenting-1-related serine/threonine kinase that regulates NKA catalytic activity by sensing changes in intracellular sodium concentration and eliciting a signaling cascade that adjusts sodium extrusion[3, 6]. Accordingly, increased intracellular sodium fuels the sodium-calcium exchange system (NCE1), prompting entrance of calcium and activation of SIK1 (Thr-322 phosphorylation) by calcium calmodulin-dependent kinase (CaMK1)[6]. SIK1 activation results in dephosphorylation of Na^+-K^+-ATPase α-subunit and increase of its maximal sodium pumping capacity via Protein Phosphatase 2A (PPA2) activation[6, 7]. Conversely, lack of salt-inducible kinase 1 is associated with increased inactivation and endocytosis of Na^+-K^+-ATPase molecules, resulting in a reduction of its activity and protein expression[6]. SIK1 is involved in short-term modulation of the activity of a selected pool of NKA molecules that can be rapidly activated for quick adjustments in sodium transport, and does not affect the basal activity responsible for the housekeeping role of the sodium pump[6]. Along with sodium-dependent signals, SIK network has been described to mediate sodium-independent signals in the modulation of active sodium transport such as dopamine, angiotensin and aldosterone, as well as being involved in mechanisms independent of ion gradient changes such as insulin action, glucose homeostasis, adipocyte energy metabolism[3, 20-27], steroid synthesis[19], cardiomyogenesis[28, 29], gene expression and transcription[30], cell polarity, intracellular junction stability and apoptosis[31-33]. Notably, salt-inducible kinase 1 has been recognized as an important factor in blood pressure regulation via distinct pathways.

Essential hypertension is a multifactorial and polygenetic condition strongly associated to high salt intake and abnormal regulation of renal sodium transport[34, 35]. Indeed, high dietary salt leads to a rise in blood pressure, left cardiac ventricular mass and arterial thickness and stiffness[35, 36]. In the renal proximal tubule, sodium uptake is driven by the sodium gradient established by the Na^+-K^+-ATPase units located at basolateral membrane[7]. Since salt-inducible

kinase 1 participates in the regulation of NKA activity under sodium stress conditions, it has been assumed that elevated basal SIK1 activity in renal epithelial cells could elicit a sustained increase in sodium reabsorption by the kidney, leading to plasma volume expansion and high blood pressure[35]. Additionally, since SIK1 has also been identified in vascular smooth muscle cells (VSMCs) and endothelial cells[37], it has been suggested that SIK1 could also comprise a major determinant in the regulation of vascular tone and development of hypertension through its influence on NKA activity in VSMCs upon variations in salt intake[37]. Recently, a single nucleotide polymorphism in the coding region of the human SIK1 gene (HapMap rs3746951) resulting in the substitution of glycine to serine (^{15}Gly→Ser) in salt-inducible kinase 1 protein has been associated with lower blood pressure and decreased left ventricular mass, a robust marker of long-term blood pressure load[37]. However, this SIK1-^{15}Ser variant strongly relates with significantly increased basal SIK1 activity[37], and a drop in sodium-calcium exchange and calcium delivery to the contractile apparatus secondary to increased Na^+-K^+-ATPase activity could in part explain the potential role of increased vascular SIK1 activity on vascular tone relaxation[38]. Moreover, it has been demonstrated in *sik1*$^{-/-}$ mice that loss of salt-induced kinase 1 induces high blood pressure and vascular remodeling processes such as dysregulated collagen synthesis and increased contractile phenotype of vascular smooth muscle cells secondary to increased endothelial production of TGF-β1, with no observed differences in renal function[39]. Therefore, it has been established that lack of SIK1 is a precipitant factor in the development of sodium-sensible hypertension due to compromised maintenance of a relaxed vascular tone under increased sodium consumption[39].

Salt-inducible kinase 1 activity has been demonstrated as necessary to prevent the development of high blood pressure under chronic high salt intake[39]. However, it is possible that SIK1 activity in other tissues besides VSMCs can also be relevant for blood pressure regulation through other unknown salt-inducible mechanisms[39]. For instance, growing evidence has been progressively proposing the gastrointestinal tract as a key element in the regulation of cardiometabolic homeostasis – besides the association of anatomical structure, gut microbiota, gut autonomic nerve activity and gut hormone secretion with blood pressure regulation, it is unanimously accepted that excessive intestinal absorption of sodium contributes to the

development of hypertension[39, 40]. Regarding these aspects, we hypothesize that SIK1 activity present in other tissues such as the gastrointestinal tract may represent an alternative pathway for blood pressure regulation under chronic high salt intake and a potentially promising target for the comprehension and management of hypertension.

The present work – in which the role of salt-inducible kinase 1 on sodium-sensible hypertension development was further explored – has two different sections.

In Part I, we characterized the effects of SIK1 ablation on blood pressure, focusing on renal and sympathetic nervous system mechanisms of salt-induced hypertension.

In Part II, we took a first step in investigating the interplay between Na^+-K^+-ATPase and basolateral K_{ATP} channels in intestinal cells, focusing on the characterization of intestinal ion transport mechanisms that are potentially subject to SIK1 regulation.

PART I

Hypertension Research
https://doi.org/10.1038/s41440-019-0249-z

ARTICLE

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

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Received: 30 April 2018 / Revised: 29 November 2018 / Accepted: 22 January 2019

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

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 homoeostasis. 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

†This study was initiated in collaboration with Dr. Alejandro M. Bertorello. Unfortunately, he passed away 23rd January 2013. All co-authors have agreed on the submission of the paper on his behalf.

Supplementary information The online version of this article (https://doi.org/10.1038/s41440-019-0249-z) contains supplementary material, which is available to authorized users.

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Published online: 20 March 2019

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 hemodynamic 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 ± 2 °C and relative humidity: 50 ± 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 200µL 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 RNAlater (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 TaqMan 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 & C^a, 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

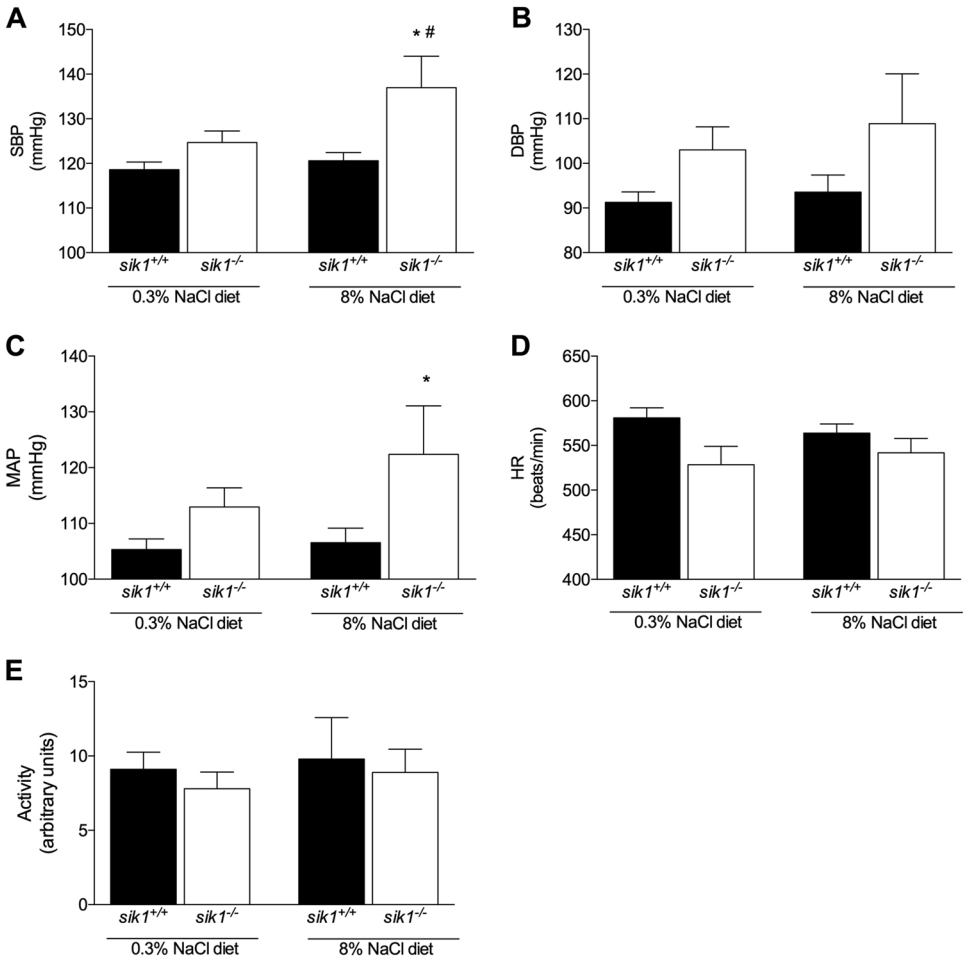

Fig. 1 Blood pressure, heart rate and home-cage activity in telemetered salt-inducible kinase 1 (SIK1) knockout (*sik1*$^{-/-}$) and wild-type (*sik1*$^{+/+}$) littermate mice challenged either a high-salt (8% NaCl) or control (0.3% NaCl) diet for 7 days. (**a**) Systolic blood pressure (SBP), (**b**) 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

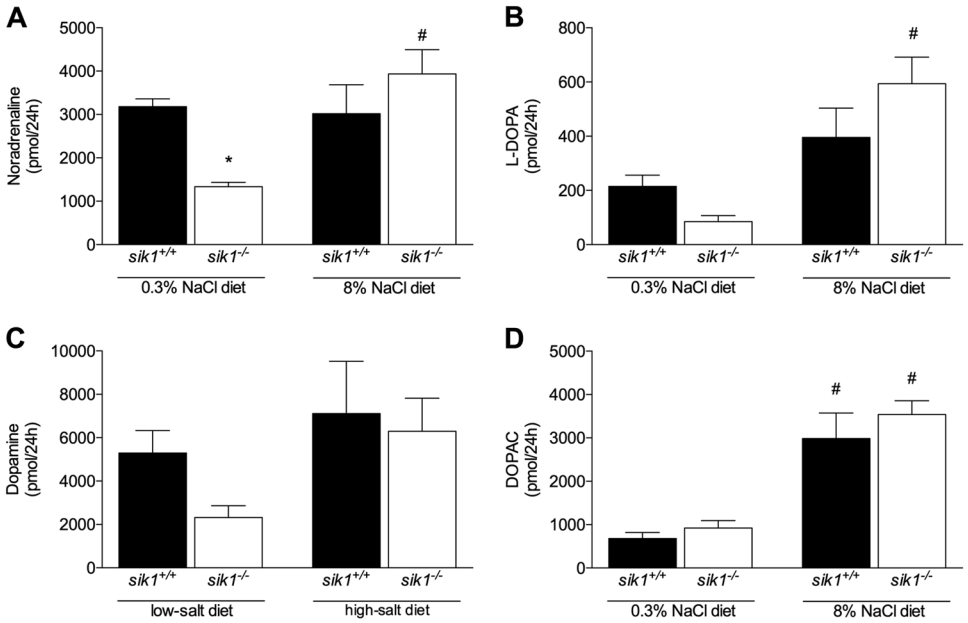

Fig. 2 Urinary L-DOPA, catecholamines and metabolites in salt-inducible kinase 1 (SIK1) knockout (*sik1*$^{-/-}$) and wild-type (*sik1*$^{+/+}$) littermate mice challenged either a high-salt (8% NaCl) or control (0.3% NaCl) diet. **a** Noradrenaline, (**b**) L-DOPA, (**c**) dopamine and (**d**) 3,4-dihydroxyphenylacetic 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

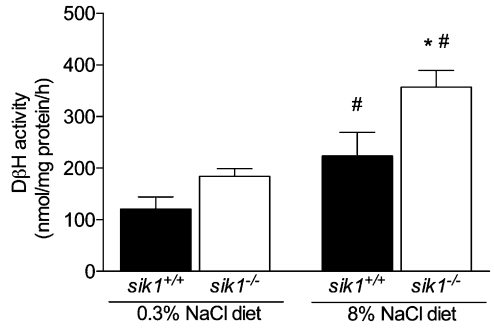

Fig. 3 Dopamine β-hydroxylase (DβH) activity in adrenal glands of salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) and wild-type ($sik1^{+/+}$) littermate mice challenged either a high-salt (8% NaCl) or control (0.3% NaCl) diet. Mean ± SEM; $n = 5$ per group; values were determined to be significantly 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

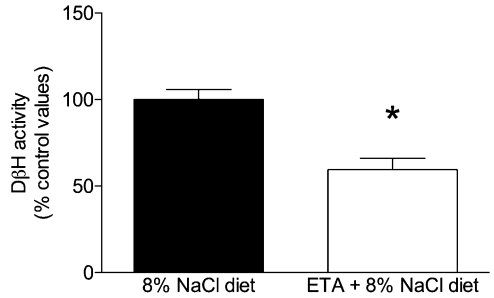

Fig. 4 Effect of etamicastat (ETA) treatment on dopamine β-hydroxylase (DβH) activity in adrenal glands of salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) mice challenged with a high-salt (8% NaCl) diet. Mean ± SEM; $n = 7$ to 8 per group; values were determined to be significantly different from 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

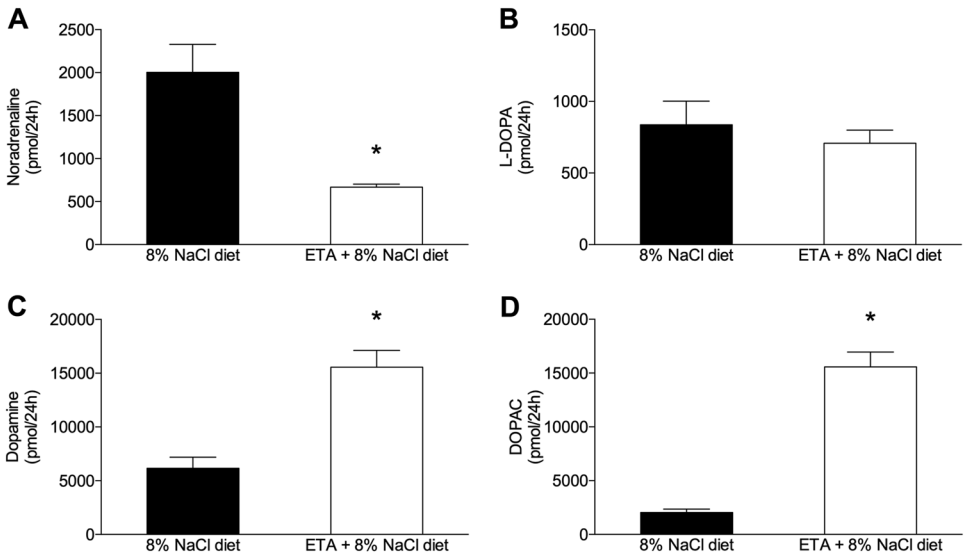

Fig. 5 Effect of etamicastat (ETA) treatment on urinary L-DOPA, catecholamines and metabolites in salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) mice challenged with a high-salt (8% NaCl) diet. **a** noradrenaline, (**b**) L-DOPA, (**c**) dopamine and (**d**) 3,4-dihydroxyphenylacetic acid (DOPAC). 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

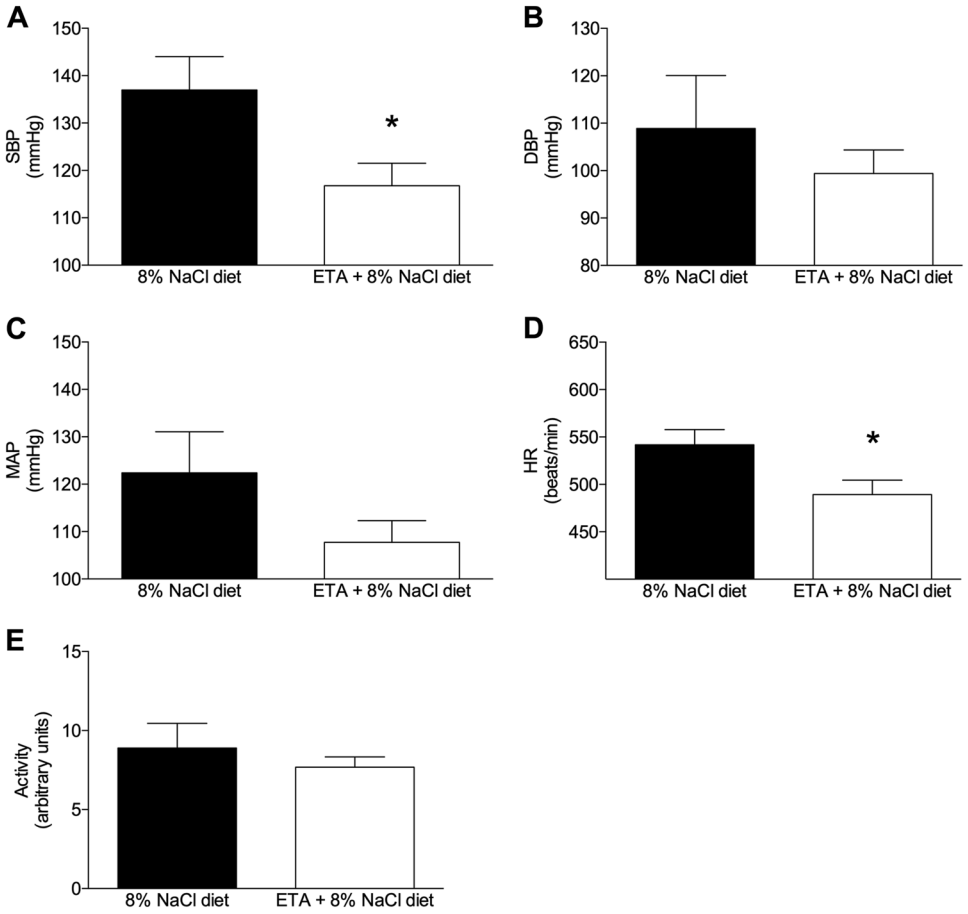

Fig. 6 Effect of 50 mg/kg/day etamicastat (ETA) treatment on blood pressure, heart rate and home-cage activity in telemetered salt-inducible kinase 1 (SIK1) knockout ($sik1^{-/-}$) mice challenged with a high-salt (8% NaCl) diet. ETA treatment for 14 days, in which in the last 7 days, animals were fed 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 noradrenargic 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 antihypertensive 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.

Acknowledgements This work was supported by Foundation for Science and Technology (FCT - Portugal, COMPETE/FEDER Grants PTDC/DTP-FTO/0735/2014 and UID/BIM/4308/2016) and BIAL-Portela & C[a], S.A.

Compliance with ethical standards

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

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PART II

INTERPLAY BETWEEN NA(+)-K(+)-ATPASE AND BASOLATERAL K(ATP) CHANNELS IN INTESTINAL ION TRANSPORT MODULATION

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Short title: Na^+-K^+-ATPase, K_{ATP} channels and intestinal ion transport

Word count: 4929

Introduction

Although numerous general mechanisms involved in sodium transcellular transport and homeostasis have been recognized in renal proximal tubule cells, the gastrointestinal tract and the kidney share substantial similarities in epithelial ion transport, as basolateral Na^+-K^+-ATPase represents the driving force for the movement of numerous substrates across cell membrane, being subject to parallel regulatory mechanisms[41-43].

During transcellular salt uptake, sodium-absorbing epithelia are recognized for their ability to match the rates of passive sodium entry across the apical membrane and active sodium extrusion across the basolateral membrane in order to maintain intracellular Na^+ concentrations constant[44]. Sodium is actively extruded from the cell by Na^+-K^+-ATPase at the expense of potassium entrance[45]. Therefore, changes in apical Na^+ entry proceed in concern with changes in Na^+-K^+-ATPase activity and thus affect K^+ uptake across the basolateral membrane[46]. Since K^+ recycling is necessary for continued sodium pump turnover, changes in NKA activity result in reciprocal adjustments in basolateral K^+ conductance, maintaining intracellular K^+ concentrations constant[46, 47]. This functional coupling between Na^+-K^+-ATPase activity and basolateral K^+ channel activity (pump-leak) is mediated by ATP-sensitive K^+ (K_{ATP}) channels[45].

K_{ATP} channels are inwardly rectifying ATP-modulated channels present in the basolateral membrane composed of four pore-forming Kir6.x subunits and four auxiliary SURx proteins[48]. These channels show an essentially voltage-independent activity that relies on intracellular nucleotides, as its opening is inhibited by adenosine triphosphate (ATP) and activated by adenosine diphosphate (ADP): stimulation of Na^+-K^+-ATPase activity results in decreased intracellular ATP levels, which in turn open the K_{ATP} channels and increase basolateral K^+ conductance[45, 47-49]. Opening of K_{ATP} channel causes membrane hyperpolarization, facilitating the K^+ recycling required for continued Na^+-K^+-ATPase functioning and Na^+ reabsorption[47].

In the second part of the present work, we took a first step in investigating the interplay between Na^+-K^+-ATPase and basolateral K_{ATP} channels in intestinal cells. Taking the previously mentioned aspects into account, along with the observation that dexamethasone treatment has been reported to increase Na^+-K^+-ATPase activity[8, 50, 51], we first characterized the changes in membrane potential of intestinal, hepatic and neuroepithelial cells exposed to drugs known to

block or open K_{ATP} channels. Next, we determined if pretreatment with dexamethasone associates with changes in membrane potential response to K_{ATP} channel blocking or opening in intestinal epithelial cells. Finally, we tested whether dexamethasone influences Na^+-K^+-ATPase protein expression and activity in these cells.

Materials and Methods

Cell Cultures

Human T84 cells (ATCC® CCL-248™), human HT-29 cells (ATCC® HTB-38™) and human CACO-2 cells (ATCC® HTB-37™), colonic adenocarcinoma cell lines, human SK-HEP1 cells (ATCC® HTB-52™), a liver adenocarcinoma cell line, and human SK-N-SH cells (ATCC® HTB-11™), a neuroblastoma-derived cell line, were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a humidified atmosphere of 5% CO_2-95% air at 37º C. T84 cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 Ham (DMEM/F-12) with 10% fetal bovine serum (Gibco 10500-064). HT-29 cells were grown in McCoy's 5A Medium with 10% fetal bovine serum (Gibco 10500-064). CACO-2 and SK-N-SH cells were grown in Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (Gibco 10500-064). SK-HEP1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 Medium with 20% fetal bovine serum (Gibco 10500-064). All growth mediums were supplemented with 10^6 U/L penicillin G, 250 μg/L amphotericin B, 100 ng/L streptomycin (Gibco 15240-062) and 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanosulfonic acid (HEPES; Sigma H3375). Cell medium was changed every 2 days and cells were dissociated with 0.25% trypsin-EDTA and subcultured in 21-cm^2 Petri dishes (Sarstedt, Germany) every 7 days.

Membrane Potential

To characterize the effects of basolateral K^+ currents and K_{ATP} channels on resting membrane potential in T84, HT-29, CACO-2, SK-HEP1 and SK-N-SH cells, changes in membrane potential were assayed after cell exposure to drugs using an anionic voltage-sensitive fluorescent dye, bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$), a slow-response probe which exhibits potential-dependent changes in cell membrane distribution accompanied by variance in fluorescence intensity – cell depolarization causes increased dye influx and enhanced fluorescence, whereas cell hyperpolarization causes dye extrusion and decreased fluorescence[52]. Used drugs are known to alter inwardly rectifying K^+ conductance through K^+ channel blocking ($BaCl_2$) or K_{ATP} channel opening (pinacidil)[52, 53]. For experiments, T84, HT-29, CACO-2, SK-HEP1 and SK-N-SH

cells were dissociated from Petri dishes with 0.25% trypsin-EDTA, plated in 96-well plates (Sarstedt, Germany) and cultured as described above. After reaching confluence, cells were rinsed twice with Krebs assay buffer (20 mM HEPES, 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 5 mM glicose; pH=7.4) containing 0.5 μM of $DiBAC_4(3)$ and incubated for 30 minutes at 37º C in order to ensure dye distribution cross cell membrane, as suggested by previous experiments[54]. After incubation, cell medium was replaced with 180 μL of Krebs assay buffer containing 0.5 μM of $DiBAC_4(3)$ and fluorescence was read for 15 minutes. Finally, 20 μL of test compounds (1 mM $BaCl_2$ and 50 μM pinacidil) were added in a 10X concentration and changes in fluorescence were monitored for 10 minutes. Fluorescence was measured at 488-nm excitation and 520-nm emission (Spectramax Gemini, Molecular Devices).

In a different subset of experiments, to test whether dexamethasone modulates the effects of basolateral K^+ currents and K_{ATP} channels on resting membrane potential, T84 cells were pretreated with varying concentrations of dexamethasone before changes in membrane potential were assayed after cell exposure to $BaCl_2$ and pinacidil. For experiments, T84 cells were dissociated from Petri dishes with 0.25% trypsin-EDTA, plated in Falcon® 96-well black/clear flat bottom TC-treated microplates (Corning, USA) in 30 000 cells/well density and cultured as described above. Cells were treated 5 days later as confluent monolayers for 48 hours with 10^{-8}, 10^{-7} and 10^{-6} M dexamethasone or vehicle (0.01% dimethyl sulfoxide – DMSO) in DMEM/F-12 medium containing 10% fetal bovine serum, as suggested by previous studies[55]. Growth medium with or without dexamethasone was replaced every day. Also, as cells exposed to dexamethasone were found to proliferate slower than untreated cells, fetal bovine serum was present in treated and control cells during the whole treatment to avoid loss of cell viability[55]. Changes in membrane potential were then assayed as described above.

In-Cell Western

To test whether dexamethasone influences the expression of Na^+-K^+-ATPase, T84 cells were dissociated from Petri dishes with 0.25% trypsin-EDTA, plated in Falcon® 96-well black/clear flat bottom TC-treated microplates (30 000 cells/well) and cultured as described above. Cells were treated 5 days later as confluent monolayers for 48 hours with 10^{-8}, 10^{-7} and 10^{-6} M dexamethasone

or vehicle (0.01% Dimethyl Sulfoxide – DMSO) in DMEM/F-12 medium containing 10% fetal bovine serum. Growth medium with or without dexamethasone was replaced every day. After treatment, cells were rinsed with TRIS buffered saline 1X (TBS 1X) and immediately fixed with 4% paraformaldehyde for 15 minutes at room temperature without shaking, as suggested by previous experiments[56]. Cells were then permeabilized twice with 0.05% Triton X-100 for 5 minutes and incubated with DyLight 800 NHS ester in phosphate-buffered saline (PBS) 1X (1:50 000 dilution; Life Technologies; catalog number 46422) for 20 minutes at room temperature with gentle shaking. After being washed thrice with PBS 1X, cells were blocked with blocking buffer (5% non-fat dry milk) for 1.5 hours at room temperature with gentle shaking. Cells were incubated with primary antibody diluted in antibody dilution buffer (2.5% non-fat dry milk) for 2 hours at room temperature with gentle shaking - NKAα1 Mouse Monoclonal Antibody (1:100 dilution; Santa Cruz; catalog number sc-21712). After being washed thrice with PBS 1X, cells were incubated with secondary antibody diluted in antibody dilution buffer for 1 hour at room temperature with gentle shaking and protected from light – Alexa Fluor 680 Goat anti-Mouse IgG (1:1000 dilution; Invitrogen; catalog number A-21056). Finally, after being washed thrice with PBS 1X, wells were imaged by scanning at 700 or 800 nm with the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln). Relative protein expression of Na^+-K^+-ATPase α1-subunit was represented by fluorescence intensity measured by image software (Odyssey Software Version 3.0, LI-COR Biosciences), normalized for DyLight 800 NHS ester-labelled proteins.

In a subsequent set of experiments, to test whether an increased time-exposure to dexamethasone affects the expression of Na^+-K^+-ATPase, T84 cells were dissociated from Petri dishes with 0.25% trypsin-EDTA, plated in Falcon® 96-well black/clear flat bottom TC-treated microplates (30 000 cells/well) and cultured as described above. Cells were treated 5 days later as confluent monolayers for 120 hours with 10^{-8}, 10^{-7} and 10^{-6} M dexamethasone or vehicle (0.01% dimethyl sulfoxide – DMSO) in DMEM/F-12 medium containing 10% fetal bovine serum. Growth medium with or without dexamethasone was replaced every day. After treatment, protocol was followed as described above. Also, to test whether an increased dose-exposure to dexamethasone changes the expression of Na^+-K^+-ATPase, T84 and HT-29 cells were dissociated from Petri dishes with 0.25% trypsin-EDTA, plated in Falcon® 96-well black/clear flat bottom TC-treated microplates

(30 000 cells/well) and cultured as described above. Cells were treated 5 days later as confluent monolayers for 48 hours with 3 μM dexamethasone or vehicle (0.01% Dimethyl Sulfoxide – DMSO) in DMEM/F-12 medium or McCoy's 5A Medium, respectively, containing 10% fetal bovine serum. Growth medium with or without Dexamethasone was replaced every day. After treatment, protocol was followed as described above for both cell lines.

Cell Viability

To evaluate the effect of dexamethasone treatment on T84 cell proliferation, cell viability was assessed using the In-Cell Western assay. DyLight 800 Amine-Reactive dye is an *N*-hydroxysuccinimide (NHS) ester moiety that reacts with exposed N-terminal α-amino or ε-amino groups of lysine residues and is used for target protein labelling for *in vitro* and *in vivo* fluorescent detection strategies. For experiments, T84 cells were dissociated from Petri dishes with 0.25% trypsin-EDTA, plated in Falcon® 96-well black/clear flat bottom TC-treated microplates (30 000 cells/well) and cultured as described above. Cells were treated 5 days later as confluent monolayers for 48 hours with 10^{-8}, 10^{-7} and 10^{-6} M dexamethasone or vehicle (0.01% dimethyl sulfoxide – DMSO) in DMEM/F-12 medium containing 10% fetal bovine serum. Growth medium with or without dexamethasone was replaced every day. After treatment, protocol was followed as described above. Cell viability was assessed based on changes in DyLight 800 NHS ester-labelled protein expression between control and treated groups.

Western Blot

To further investigate whether dexamethasone influences the expression of Na^+-K^+-ATPase, T84 and HT-29 cells were dissociated from Petri dishes with 0.25% trypsin-EDTA, plated in 6-well plates (Sigma-Aldrich, USA) and cultured with growth medium for 24 hours after seeding. For experiments, cells were then treated for 48 hours with 1 and 10 μM dexamethasone or vehicle (0.01% dimethyl sulfoxide – DMSO) in growth medium containing 10% fetal bovine serum and further kept in growth medium for 24 hours in order to reach confluence. After treatment, cells were rinsed twice with TBS 1X and exposed to RIPA Lysis Buffer (150 mM NaCl, 50 mM Tris-HCl pH=7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) containing

protease inhibitors (1 mM PMSF, 1 μL/mL of aprotinin and 1 μL/mL of leupeptin) and phosphatase inhibitors (5 mM Na_3VO_4 and 5 mM NaF). Cells were scraped from 6-well plates, lysed by sonication (40 Hz; 10 seconds) and incubated on ice for 30 minutes, in order to obtain total protein extraction. After centrifugation (13 200 g for 30 minutes at 4º C), the supernatants were collected and the amount of protein was quantified using the method of Bradford (Bio-Rad). Proteins (30 μg) were warmed at 70º C for 10 minutes, separated on a 7.5% SDS-polyacrylamide (2 hours; 140 mV) and electrotransfered to nitrocellulose membranes. Membranes were blocked in blocking buffer (5% non-fat dry milk) for 1 hour at room temperature with gentle shaking. After being washed with TBS-T, membranes were incubated overnight with primary antibodies diluted in antibody dilution buffer (2.5% non-fat dry milk) at 4º C – NKAα1 Mouse Monoclonal Antibody (1:1000 dilution; Santa Cruz; catalog number sc-21712) and GAPDH Mouse Monoclonal Antibody (1:20 000 dilution; Santa Cruz; catalog number sc-32233). After being washed four times with TBS-T, membranes were incubated with secondary antibody diluted in antibody dilution buffer for 1 hour at room temperature with gentle shaking and protection from light – Alexa Fluor 680 Goat anti-Mouse IgG (1:1000 dilution; Invitrogen; catalog number A-21056). Finally, after being washed four times with TBS-T and once with TBS, membranes were imaged by scanning at 700 or 800 nm with the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln). Relative abundance of Na^+-K^+-ATPase α1-subunit was represented by fluorescence intensity measured by image software (Odyssey Software Version 3.0, LI-COR Biosciences), normalized for GAPDH.

Fluorometric Assay

To investigate whether dexamethasone influences Na^+-K^+-ATPase activity, T84 cells were plated in 21-cm^2 Petri dishes (Sarstedt, Germany) and cultured as described above until 2 days after reaching confluence. For experiments, cells were treated for 48 hours with 10^{-6} M dexamethasone or vehicle (0.01% dimethyl sulfoxide – DMSO) in DMEM/F-12 medium containing 10% fetal bovine serum. Growth medium with or without dexamethasone was replaced every day. Cells were then detached with 0.25% trypsin-EDTA and resuspended in Buffer A (10 nM HEPES and 250 mM sucrose; ph=7.4) containing protease inhibitors (0.2 μL/mL of aprotinin and 0.2 μL/mL of leupeptin), lysed by sonication (40 Hz; 10 seconds) and incubated at 30º C for 10 minutes with 0.3

mg/mL sodium dodecyl sulfate (SDS) in order to obtain total protein extraction. Next, cell lysates were resuspended in Buffer A and the amount of protein was quantified using the method of Bradford (Bio-Rad). The activity of Na^+-K^+-ATPase was then measured by fluorometric method using 3-*O*-methylfluorescein phosphate (3-*O*-MFP). This compound is a substrate for the phosphatase reaction catalyzed by Na^+-K^+-ATPase and a precursor of the fluorescent compound 3-*O*-methylfluorescein (3-*O*-MF), thereby exhibiting Na^+-K^+-ATPase activity-dependent variance in fluorescence intensity – higher NKA activity results in increased cleavage of the phosphate group from 3-*O*-MFP and formation of 3-*O*-MF, thus enhancing fluorescence emission[57, 58]. As suggested by previous reports[58], the experiment was performed in assay medium containing 5 mM creatine phosphate, 4 mM $MgCl_2$, 0.5 mM EGTA, 80 mM Tris-HCl and 50 μM 3-*O*-MFP. Baseline fluorescence was assessed for 60 seconds, before 10 μL of cell lysates (~1 mg of protein) were added and fluorescence was recorded over a 140-second period. Then, 10 μL of 2 M KCl (final concentration of 10 mM) were added to activate Na^+-K^+-ATPase and fluorescence was monitored for additional 200 seconds. Finally, experiments were repeated for assay medium containing 5 mM ouabain. As K^+ is necessary for pump activation and ouabain is a known inhibitor of ATP-dependent sodium potassium exchange[58, 59], the activity of Na^+-K^+-ATPase was calculated as the slope difference in fluorescence recordings before and after addition of K^+, with and without ouabain in the buffer. These values were then related to protein content of cell lysates and final expression of activity was given as nanomoles of liberated phosphate per milligram of protein per minute (nmol P_i/mg/min). Fluorescence was measured at 470-nm excitation and 510-nm emission (FluoroMax-2, Jobin Yvon-Spex Instruments).

Data Analysis

Arithmetic means are given with standard deviation and geometrical means are given with 95% confidence values. Statistical analysis was performed using One-Way analysis of variance (ANOVA), followed by Student's *t* test or Newman-Keuls test for multiple comparisons in GraphPad Prism (GraphPad Software, San Diego). A *P*-value<0.05 was assumed to denote a significant difference. Graphs were prepared using GraphPad Prism (GraphPad Software, San Diego) and error bars depict SEM unless otherwise noted.

Drugs

$BaCl_2$ and KCl were obtained from Merk (Darmstadt, Germany). Pinacidil, dexamethasone, ouabain and DMEM/F-12, McCoy's 5A, MEM and RPMI 1640 mediums were purchased from Sigma-Aldrich (St. Louis, USA). Fetal bovine serum and antibiotic were obtained from Thermo Fisher Scientific (California, USA).

Results

Effects of basolateral K^+ currents and K_{ATP} channels on resting membrane potential in human T84, HT-29, CACO-2, SK-HEP1 and SK-N-SH cells

To characterize how modification of basolateral K^+ conductance affects resting membrane potential, we used the voltage-sensitive dye $DiBAC_4(3)$ to assess changes in membrane potential in human T84, HT-29, CACO-2, SK-HEP1 and SK-N-SH cells following the application of drugs that inhibit or stimulate these ionic currents through ATP-sensitive K^+ (K_{ATP}) channels. Results are shown in Figure 1. We observed an increase in $DiBAC_4(3)$ fluorescence after exposure of T84 cells to 1 mM Ba^{2+}, indicating a depolarization (Figure 1*A*). Conversely, fluorescence intensity decreased after exposure of T84 cells to 50 μM pinacidil, indicating a hyperpolarization (Figure 1*A*). This pattern of T84 cell depolarization and hyperpolarization following application of 1 mM BaCl and 50 μM pinacidil, respectively, was similar to the pattern observed in HT-29, CACO-2, SK-HEP1 and SK-N-SH cells under the same experimental conditions (Figure 1, *B-E*).

Effects of dexamethasone on basolateral K^+ currents and K_{ATP} channels in human T84 cells

To assess whether dexamethasone modulates the effects of basolateral K^+ currents and K_{ATP} channels on resting membrane potential, human T84 cells were pretreated for 48 hours with varying concentrations of dexamethasone (10^{-8}, 10^{-7} and 10^{-6} M) and changes in cell membrane potential after treatment with $BaCl_2$ and pinacidil were monitored with the use of fluorescent dye $DiBAC_4(3)$. Results are shown in Figure 2. In control group, mean change in fluorescence after treatment with 1 mM $BaCl_2$ and 50 μM pinacidil was 549.5 ± 50.13 (95% confidence interval of 451.2 to 647.8) and -63.56 ± 35.45 (95% confidence interval of 0 to -133), respectively. In 10^{-8} M dexamethasone treatment group, mean change in fluorescence after treatment with 1 mM $BaCl_2$ and 50 μM pinacidil was 695.1 ± 28.03 (95% confidence interval of 640.2 to 750) and -3.183 ± 2.092 (95% confidence interval of 0 to -7.283), respectively. In 10^{-7} M dexamethasone treatment group, mean change in fluorescence after treatment with 1 mM $BaCl_2$ and 50 μM pinacidil was 427.8 ± 28.79 (95% confidence interval of 371.4 to 484.3) and -207 ± 13.5 (95% confidence interval of -180.6 to -233.5), respectively. In 10^{-6} M dexamethasone treatment group, mean change in

fluorescence after treatment with 1 mM $BaCl_2$ and 50 μM pinacidil was 352 ± 45.43 (95% confidence interval of 264 to 442) and -346 ± 25.55 (95% confidence interval of -296.3 to -396.5), respectively. Treatment with 10^{-7} and 10^{-6} M dexamethasone led to a significant reduction in $DiBAC_4(3)$ fluorescence after exposure of T84 cells to 1 mM $BaCl_2$ and 50 μM pinacidil, indicating a decreased depolarization response to K_{ATP} channel blocking (mean differences in fluorescence emission of -121.7 ± 19.67 and -196.5 ± 19.67, respectively) and an increased hyperpolarization response to K_{ATP} channel opening (mean differences in fluorescence emission of -143.4 ± 11.45 and -282.8 ± 11.45, respectively). However, at minimal concentrations (10^{-8} M), dexamethasone treatment leads to a significant rise in $DiBAC_4(3)$ fluorescence after exposure of T84 cells to 1 mM $BaCl_2$ and 50 μM pinacidil, indicating an increased depolarization response to K_{ATP} channel blocking (mean difference in fluorescence emission of 145.6 ± 19.67) and an decreased hyperpolarization response to K_{ATP} channel opening (mean difference in fluorescence emission of 60.38 ± 11.45).

Effects of dexamethasone on Na^+-K^+-ATPase protein expression and cell viability in human T84 and HT-29 cells

To examine whether the dexamethasone-induced modulation of basolateral K^+ conductance is mediated by altered Na^+-K^+-ATPase protein expression, human T84 cells were exposed for 48 hours to varying concentrations of dexamethasone (10^{-8}, 10^{-7} and 10^{-6} M) and subjected to In-Cell Western analysis to evaluate changes in Na^+-K^+-ATPase α1-subunit abundance. Results are shown in Figure 3*A* – for 48-hour exposure, mean protein expression in the control, 10^{-8}, 10^{-7} and 10^{-6} M dexamethasone treatment groups was 0.1072 ± 0.01918 (95% confidence interval of 0.1232 to 0.09112), 0.1049 ± 0.01571 (95% confidence interval of 0.118 to 0.09176), 0.1256 ± 0.02237 (95% confidence interval of 0.1443 to 0.1069) and 0.1108 ± 0.0185 (95% confidence interval of 0.1263 to 0.09537), respectively. The relative abundance of Na^+-K^+-ATPase was not significantly altered following treatment. Alongside, the effects of dexamethasone on T84 cell proliferation were also evaluated. As depicted in Figure 4, T84 cell proliferation was not significantly affected by 10^{-8}, 10^{-7} and 10^{-6} M dexamethasone exposure – 93.83 ± 7.584% of control, 84.09 ± 7.584% of control and 86.67 ± 7.584% of control, respectively.

To test whether an increased time-exposure to dexamethasone induces a different response in Na^+-K^+-ATPase protein expression, human T84 cells were exposed for 120 hours to the same concentrations of dexamethasone (10^{-8}, 10^{-7} and 10^{-6} M) and subjected to In-Cell Western analysis to evaluate changes in Na^+-K^+-ATPase α1-subunit protein abundance. Results are shown in Figure 3*B* – for 120-hour exposure, mean relative protein expression in the control, 10^{-8} M, 10^{-7} M and 10^{-6} M dexamethasone treatment groups was 0.085 $\pm$ 0.01077 (95% confidence interval of 0.094 to 0.076), 0.097 $\pm$ 0.01254 (95% confidence interval of 0.1075 to 0.08652), 0.1011 $\pm$ 0.02231 (95% confidence interval of 0.1198 to 0.08248) and 0.09013 $\pm$ 0.01229 (95% confidence interval of 0.1004 to 0.07985), respectively. The relative abundance of Na^+-K^+-ATPase was not significantly altered following treatment.

To test whether an increased dose-exposure to dexamethasone causes a change in Na^+-K^+-ATPase protein expression, human T84 and HT-29 cells were exposed for 48 hours with 3 μM dexamethasone and subjected to In-Cell Western analysis to evaluate changes in Na^+-K^+-ATPase α1-subunit protein abundance. Results are shown in Figure 5. For T84 cells, mean relative protein expression in the control and treatment groups was 5.015 $\pm$ 0.5805 (95% confidence interval of 4.53 to 5.501) and 6.506 $\pm$ 0.5533 (95% confidence interval of 6.044 to 6.969), respectively (Figure 5*A*). For HT-29 cells, mean relative protein expression in the control and treatment groups was 4.955 $\pm$ 1.423 (95% confidence interval of 3.765 to 6.114) and 6.955 $\pm$ 1.765 (95% confidence interval of 5.48 to 8.431), respectively (Figure 5*B*). The relative abundance of Na^+-K^+-ATPase was significantly higher following treatment (mean difference of 1.491 $\pm$ 0.2835 and 2 $\pm$ 0.8015, respectively).

Human T84 and HT-29 cells were also subjected to Western Blot analysis to further test whether higher concentrations of dexamethasone (1 and 10 μM) elicit an increase in Na^+-K^+-ATPase α1-subunit protein abundance during a 48-hour treatment. Results are shown in Figure 6. For T84 cells, mean relative protein expression in the control, 1 and 10 μM dexamethasone treatment groups was 0.2655 $\pm$ 0.005931 (95% confidence interval of 0.2122 to 0.3188), 0.2373 $\pm$ 0.009395 (95% confidence interval of 0.1529 to 0.3217) and 0.2905 $\pm$ 0.0007128 (95% confidence interval of 0.2841 to 0.2969), respectively (Figure 6*A*). For HT-29 cells, mean relative protein

expression in the control, 1 and 10 μM dexamethasone treatment groups was 0.05154 ± 0.007813 (95% confidence interval of -0.01866 to 0.1217), 0.03545 ± 0.001595 (95% confidence interval of 0.02113 to 0.04978),and 0.09755 ± 0.01479 (95% confidence interval of -0.03532 to 0.2304), respectively (Figure 6*B*). As shown, 48-hour treatment with 10 μM dexamethasone prompts an upregulation of Na^+-K^+-ATPase in T84 cells (mean difference of 0.02503 ± 0.006428), with consistent findings for HT-29 cells (mean difference of 0.04602 ± 0.009701).

Effects of dexamethasone on Na^+-K^+-ATPase activity in human T84 cells

To verify whether dexamethasone regulates the activity of Na^+-K^+-ATPase, human T84 cells were exposed for 48 hours to 10^{-6} M dexamethasone and subjected to fluorometric assay using 3-*O*-methylfluorescein phosphate (3-*O*-MFP) to measure NKA activity. We considered the slope difference in fluorescence recordings before and after addition of K^+ to assay medium, with and without ouabain in the buffer, as an indirect measure of NKA activity, which was related to protein content of cell lysates and thus converted to nanomoles of liberated phosphate per milligram of protein per minute (nmol P_i/mg/min). Results are shown in Figure 7. Mean Na^+-K^+-ATPase activity in the control and treatment groups was 12.50 ± 1.578 (95% confidence interval of 11.38 to 13.61) and 14.92 ± 3.530 (95% confidence interval of 12.42 to 17.41), respectively. Mean Na^+-K^+-ATPase activity was slightly higher following treatment (mean difference of 2.423 ± 2.734).

Discussion

The aim of present experimental study was to investigate the functional interplay between Na^+-K^+-ATPase activity and basolateral K^+ channel activity in intestinal cells, a core mechanism in sodium uptake extensively considered in renal proximal tubule models. In our results, a modulation of basolateral K^+ conductance has been observed after incubation of human T84 cells with low concentrations of dexamethasone (10^{-7} and 10^{-6} M) without accompanying increases in Na^+-K^+-ATPase protein expression. Increased Na^+-K^+-ATPase activity was also seen in these cells following treatment. These effect are in agreement with the view that dexamethasone-induced stimulation of Na^+-K^+-ATPase activity associates with an increased basolateral K^+ channel activity as a result of the pump-leak coupling mechanism required for continued transcellular sodium absorption. Furthermore, our results show for the first time that higher concentrations of dexamethasone (3 and 10 μM) produce an upregulation of Na^+-K^+-ATPase in intestinal cells.

We first started to characterize how modification of basolateral K^+ conductance through ATP-sensitive K^+ (K_{ATP}) channels affects resting membrane potential in different intestinal and non-intestinal cell lines. K_{ATP} channel activity has been demonstrated to be dependent of intracellular nucleotides: low intracellular ATP levels disinhibit K_{ATP} channels, resulting in increased basolateral K^+ conductance and membrane hyperpolarization[48, 49]. As barium (Ba^{2+}) is a inwardly rectifying potassium (K_{ir}) channel blocker and pinacidil is a K_{ATP} channel opener, we expected a change in cell membrane potential in the presence of these compounds[52, 53]. Using the voltage-sensitive dye $DiBAC_4(3)$, our results demonstrate that altering basolateral K^+ conductance through ATP-sensitive K^+ (K_{ATP}) channels can lead to changes in cell membrane potential in T84, HT-29, CACO-2, SK-HEP1 and SK-N-SH cells; K_{ATP} channel blocking by $BaCl_2$ causes cell depolarization, whereas K_{ATP} channel opening by pinacidil causes cell hyperpolarization.

In a second set of experiments, we explored the relationship between K_{ATP} channels and cellular metabolism secondary to Na^+-K^+-ATPase activity in intestinal cells. It is well established in renal proximal tubule cells that coordination between Na^+-K^+-ATPase activity and basolateral K^+ channel conductance (pump-leak coupling) is an important functional mechanism for cell volume and homeostasis during epithelial transport[45]. Stimulation of NKA activity leads to increased K^+ conductance, which appears to result from the opening of K_{ATP} channels in the basolateral

membrane after local reductions in ATP levels[60]. It has been recognized that dexamethasone enhances Na^+-K^+-ATPase activity[8, 50, 51]. Using the $DiBAC_4(3)$ probe, we demonstrate that 48-hour dexamethasone pretreatment causes a concentration-dependent modulation of basolateral K^+ conductance and K_{ATP} channels activity in T84 cells, thus leading to significant changes in membrane potential response after exposure to $BaCl_2$ and pinacidil. Dexamethasone promoted a decreased depolarization response to K_{ATP} channel blocking and an increased hyperpolarization response to K_{ATP} channel opening at concentrations of 10^{-7} and 10^{-6} M, but not at 10^{-8} M. Besides increasing basolateral ATP-sensitive K^+ conductance, higher NKA activity in intestinal cells may directly affect resting membrane potential, resulting in membrane hyperpolarization[47]. By applying a depolarizing or hyperpolarizing stimulus in a progressively hyperpolarized membrane, K_{ATP} channel blocking results in progressively smaller overshoots and K_{ATP} channel opening results in progressively larger undershoots. Since the primary role of K_{ATP} channels is to allow recycling of K^+ pumped into the cell by Na^+-K^+-ATPase, mediating hyperpolarization of the basolateral membrane[45], these results suggest the interpretation that dexamethasone-induced changes in basolateral K^+ currents and K_{ATP} channel activity may be secondary to increased Na^+-K^+-ATPase activity.

As an increased Na^+-K^+-ATPase activity can reflect either an upregulation of this enzyme or a boosted catalytic activity of the NKA units present at the plasma membrane, we first tested whether the dexamethasone-induced modulation of basolateral K^+ currents was mediated by increased Na^+-K^+-ATPase protein expression. Using the In-Cell Western analysis, our results showed no significant increase in Na^+-K^+-ATPase abundance in T84 cells under the same experimental conditions. Later, we verified whether dexamethasone regulates NKA activity at concentrations associated with peak effects in basolateral K^+ conductance modulation. Using the 3-*O*-methylfluorescein phosphate (3-*O*-MFP) fluorometric assay, a rise in Na^+-K^+-ATPase activity was observed in T84 cells treated with 10^{-6} M dexamethasone. Therefore, we demonstrate that dexamethasone produces changes in basolateral ATP-sensitive K^+ conductance that are independent of Na^+-K^+-ATPase upregulation and most likely attributable to increased NKA activity. However, since Dexamethasone is known to induce a number of cellular changes in cultured human cells in a time- and concentration-dependent fashion, we cannot exclude the possibility

that other ion transport mechanisms such as sodium-potassium-chloride (Na-K-Cl) cotransporter[55], chloride (Cl^-) channels[61], potassium (K^+) channels[62] and sodium-potassium (Na/K) antiporter [63] could also be affected and thus be relevant for the corticoid-induced modulation of basolateral K^+ currents. Hence, future experiments using the Ussing Chamber Technique to measure the effects of dexamethasone on Na^+-K^+-ATPase activity and basolateral membrane K^+ conductance would provide more accurate information on the contribution of other intestinal ion transporter systems.

In a subsequent set of experiments, we wanted to investigate whether an increased time- and dose-exposure to dexamethasone would induce changes in Na^+-K^+-ATPase expression. Using In-Cell Western analysis, our results did not show significant differences in NKA abundance in T84 cells exposed to 10^{-8} M, 10^{-7} M and 10^{-6} M dexamethasone after extending the treatment period to 120 hours. On the other hand, a significant rise in NKA protein expression was seen in T84 cells treated for 48 hours with 3 μM dexamethasone. In fact, the same upregulating response was observed in HT-29 cells, an additional intestinal epithelial cell line. Moreover, by using a different method for protein quantification in Western Blot, we also confirmed a dexamethasone-induced sodium pump overexpression in T84 and HT-29 cells at an even higher concentration of dexamethasone (10 μM).

Taken together, our results are in agreement with the view that dexamethasone treatment leads to a concentration-dependent modulation of basolateral K^+ currents that is independent of Na^+-K^+-ATPase protein overexpression and most likely secondary to increased NKA activity, as a result of the functional coupling between Na^+-K^+-ATPase and basolateral K_{ATP} channels present in intestinal cells. Furthermore, we also report for the first time that dexamethasone can induce an upregulation of Na^+-K^+-ATPase in intestinal epithelial cells at higher treatment concentrations.

Acknowledgments

This work was supported by Foundation for Science and Technology (FCT – Portugal, COMPETE/FEDER Grants).

Conflict of Interest

The authors declare no conflict of interest.

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FIGURES AND FIGURE LEGENDS

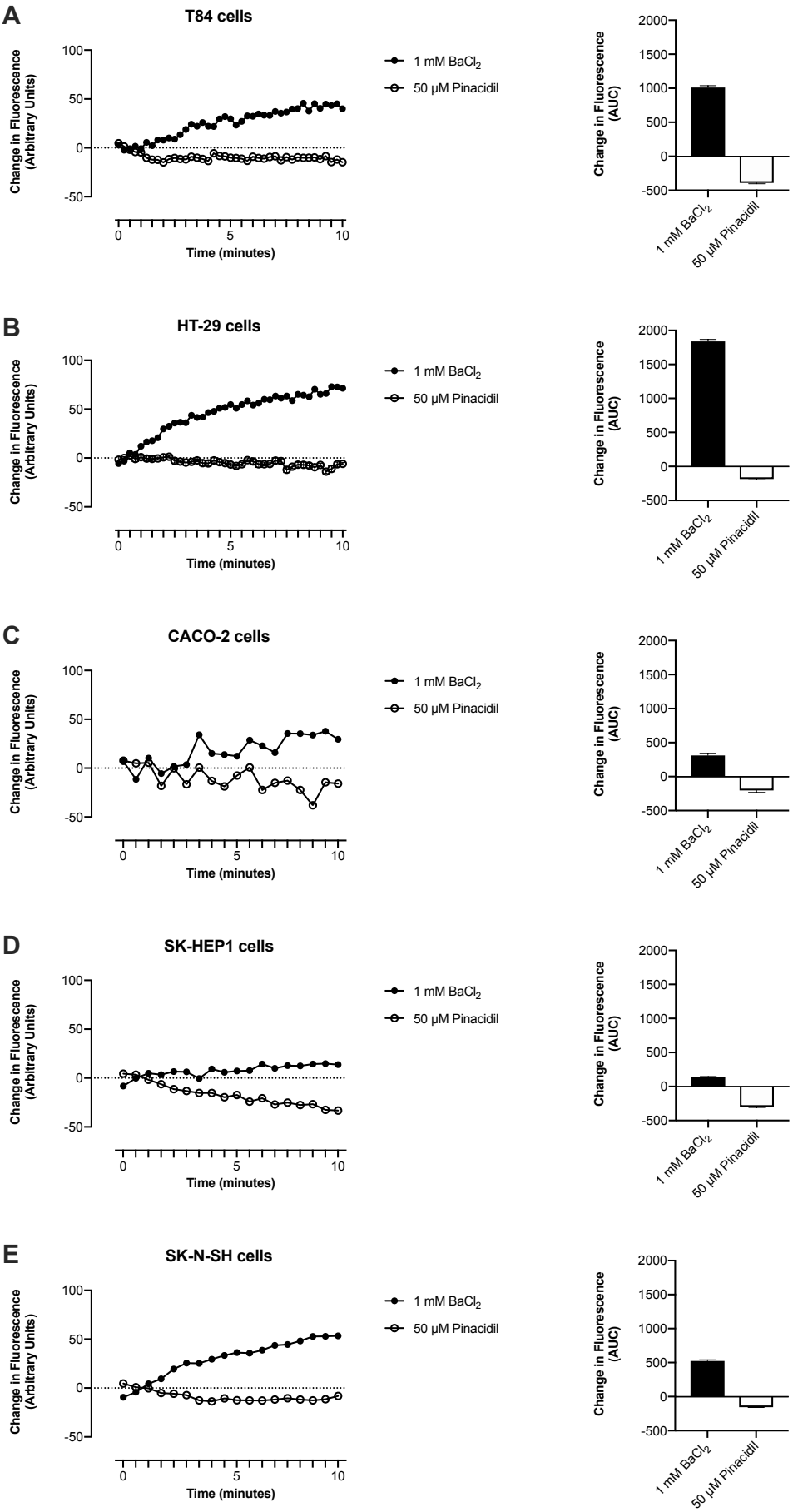
A
T84 cells
B
HT-29 cells
C
CACO-2 cells
D
SK-HEP1 cells
E
SK-N-SH cells
Change in Fluorescence (Arbitrary Units)
Time (minutes)
1 mM BaCl2
50 μM Pinacidil
Change in Fluorescence (AUC)
1 mM BaCl2
50 μM Pinacidil

Figure 1. Effects of basolateral K^+ currents and K_{ATP} channels on resting membrane potential in human T84 (**A**), HT-29 (**B**), CACO-2 (**C**), SK-HEP1 (**D**) and SK-N-SH cells (**E**). Plotted symbols show values for the average of cells (*n*=8 for A-B; *n*=4 for D-E). Traces and summarized data (area under the curve) show time course-dependent changes in $DiBAC_4(3)$ fluorescence emission after cell exposure to 1 mM $BaCl_2$ and 50 μM pinacidil, indicating that K_{ATP} channel blocking by Ba^{2+} leads to cell depolarization, whereas K_{ATP} channel opening by pinacidil leads to cell hyperpolarization in all tested cell lines. Fluorescence was read for 10 minutes and assay buffer contained 0.5 μM $DiBAC_4(3)$ in all experiments.

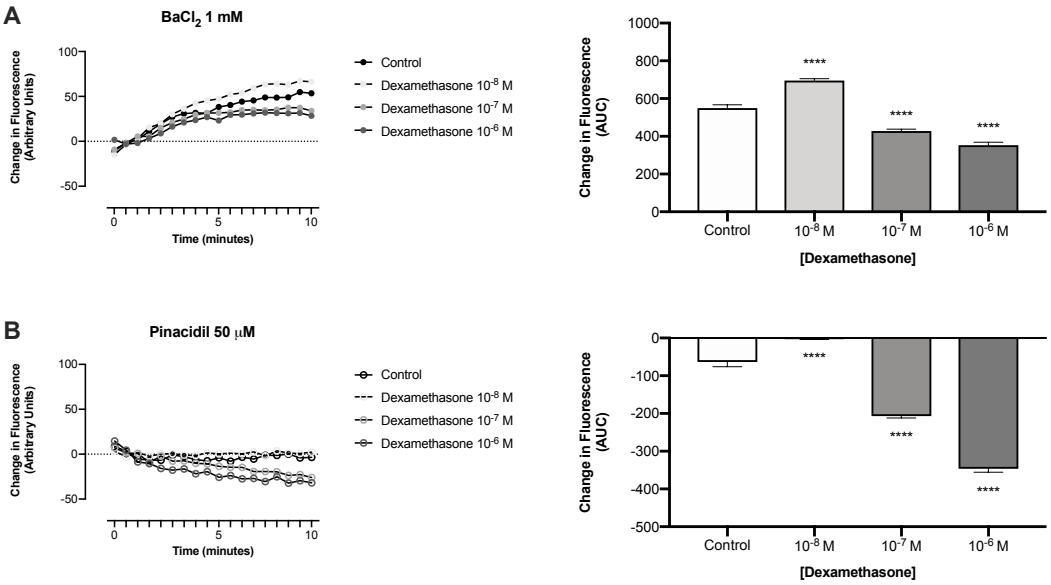

Figure 2. Concentration-dependent effects of dexamethasone on basolateral K^+ currents and K_{ATP} channels in human T84 cells. Summarized data shows changes in $DiBAC_4(3)$ fluorescence emission after exposure of T84 cells pretreated for 48 hours with varying concentrations of dexamethasone or vehicle to 1 mM $BaCl_2$ (**A**) and 50 μM pinacidil (**B**). Columns show mean values and vertical lines indicate SEM (*n*=8). Treatment with 10^{-8} M dexamethasone leads to increased depolarization response to K_{ATP} channel blocking and decreased hyperpolarization response to K_{ATP} channel opening, whereas treatment with 10^{-7} and 10^{-6} M dexamethasone leads to decreased depolarization response to K_{ATP} channel blocking and increased hyperpolarization response to K_{ATP} channel opening.

****Significant differences from control group (*P*-value<0.0001).

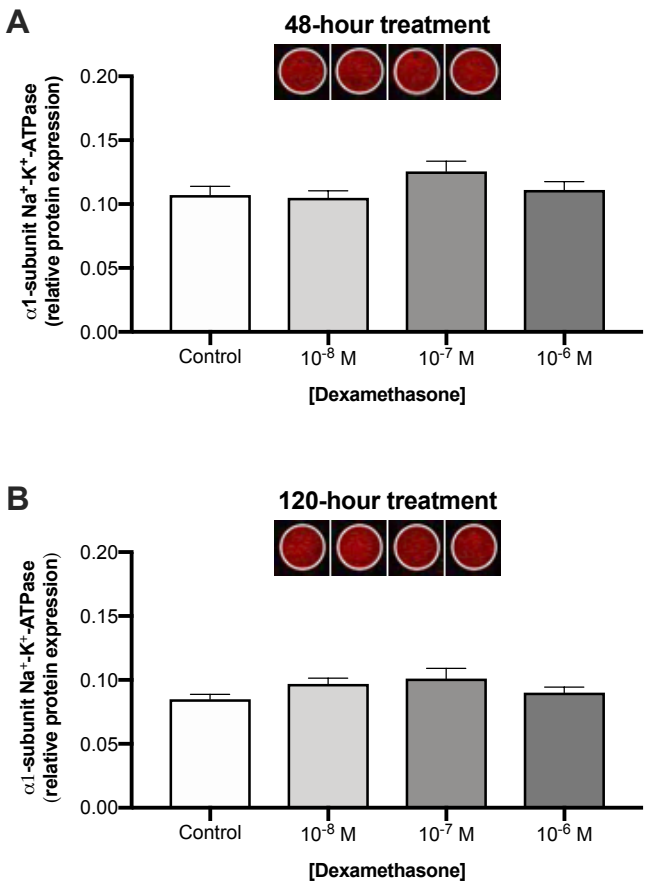

Figure 3. Effects of dexamethasone on Na^+-K^+-ATPase protein expression in human T84 cells. Relative abundance of Na^+-K^+-ATPase α1-subunit in T84 cells treated for 48 hours (**A**) and 120 hours (**B**) with varying concentrations of dexamethasone or vehicle, determined by In-Cell Western analysis. Columns show mean values and vertical lines indicate SEM (*n*=8). Protein expression was normalized for DyLight 800 NHS ester-labelled proteins. Treatment for 48 and 120 hours with 10^{-8}, 10^{-7} and 10^{-6} M dexamethasone does not show significant differences in Na^+-K^+-ATPase α1-subunit expression in T84 and HT-29 cells.

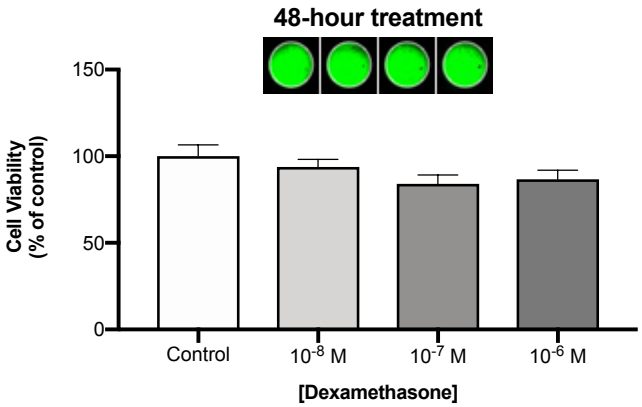

Figure 4. Effects of dexamethasone on human T84 cell viability. Summarized graphics show percentage abundance of DyLight 800 NHS ester-labelled proteins in T84 cells treated for 48 hours with varying concentrations of dexamethasone in relation to control, determined by In-Cell Western analysis. Columns show mean values and vertical lines indicate SEM (*n*=8). Treatment for 48 hours with 10^{-8}, 10^{-7} and 10^{-6} M dexamethasone does not show significant differences in T84 cell proliferation.

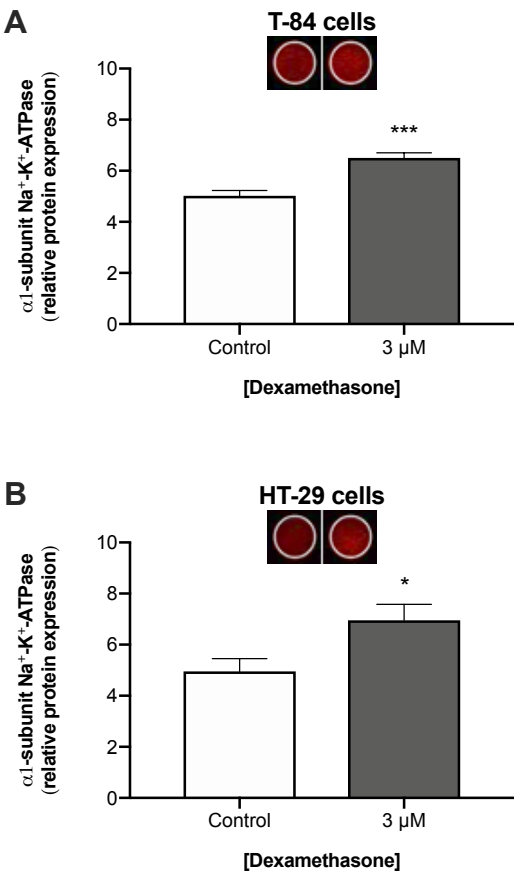

Figure 5. Effects of dexamethasone on Na^+-K^+-ATPase protein expression in human T84 and HT-29 cells. Relative abundance of Na^+-K^+-ATPase α1-subunit in T84 (**A**) and HT-29 cells (**B**) treated for 48 hours with 3 μM dexamethasone or vehicle, determined by In-Cell Western analysis. Columns show mean values and vertical lines indicate SEM (*n*=8). Protein expression was normalized for DyLight 800 NHS ester-labelled proteins. Treatment for 48 hours with 3 μM dexamethasone leads to a significant upregulation of Na^+-K^+-ATPase α1-subunit expression in T84 and HT-29 cells.

* Significant differences from control group (*P*-value<0.05).

*** Significant differences from control group (*P*-value<0.005).

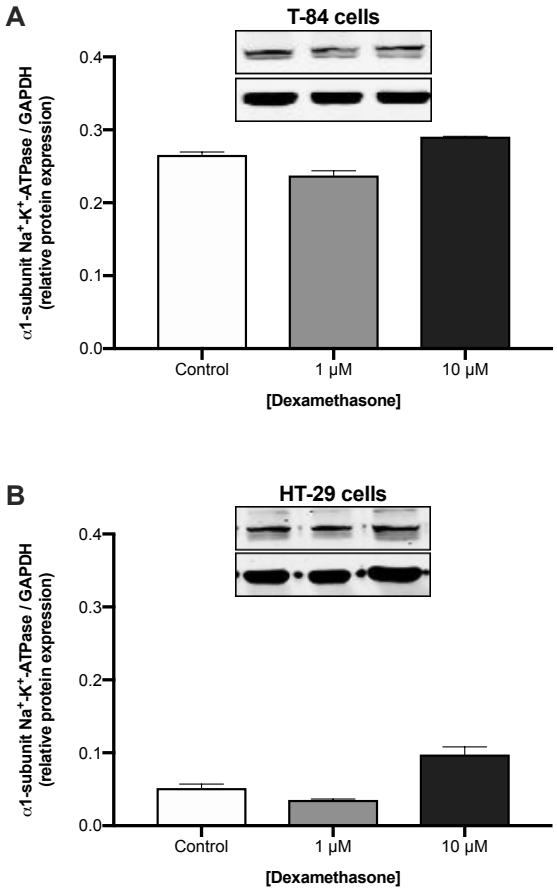

Figure 6. Effects of dexamethasone on Na^+-K^+-ATPase protein expression in human T84 and HT-29 cells. Relative abundance of Na^+-K^+-ATPase α1-subunit in T84 (**A**) and HT-29 cells (**B**) treated for 48 hours with 1 and 10 μM dexamethasone or vehicle, determined by Western Blot analysis. Columns show mean values and vertical lines indicate SEM (*n*=2). Protein expression was normalized for GAPDH. Treatment for 48 hours with 10 μM dexamethasone leads to upregulation of Na^+-K^+-ATPase α1-subunit expression in T84 and HT-29 cells.

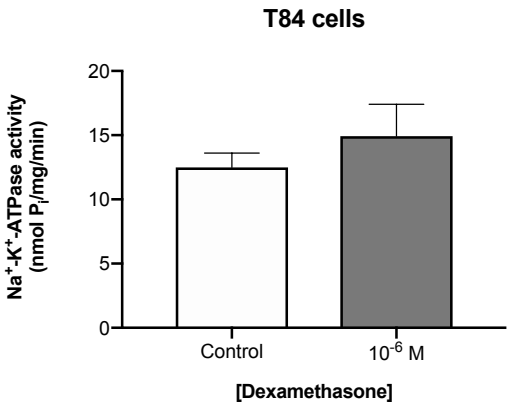

Figure 7. Effects of dexamethasone on Na^+-K^+-ATPase activity in human T84 cells. Summarized data shows the ouabain-sensitive NKA activity in T84 cells pretreated for 48 hours with 10^{-6} M dexamethasone, determined by the 3-*O*-MFP fluorometric method. Columns show mean values and vertical lines indicate SEM (*n*=2). Treatment with 10^{-6} M dexamethasone leads to increased Na^+-K^+-ATPase activity in T84 cells.

General Discussion

Despite being a multifactorial and polygenetic condition strongly associated to high salt intake, the pathogenesis of hypertension remains obscure and requests for continued research on behalf of better understanding of the disease and development of new therapeutic strategies[34, 35, 40]. Recently, salt-inducible kinase 1 (SIK1) has been recognized as an important factor in blood pressure regulation via modulation of Na^+-K^+-ATPase (NKA) activity under sodium stress conditions[6]. In particular, changes in renal and vascular SIK1 and NKA activities have been described in association with the development of salt-sensitive hypertension[35, 39]. On the other hand, the gastrointestinal tract has also been suggested as a core element in the regulation of cardiometabolic homeostasis, since excessive intestinal sodium uptake is recognized to encourage the development of hypertension[40]. Regarding these aspects, we hypothesized that SIK1 activity present in other tissues such as the gastrointestinal tract might represent an alternative salt-inducible mechanism for blood pressure regulation under chronic high salt intake, rendering a promising target for the comprehension and management of hypertension.

The present work further studied the role of SIK1 in sodium-sensible hypertension development in two sections. In Part I, we have characterized the effects of SIK1 ablation on blood pressure, focusing on renal and sympathetic nervous system mechanisms of salt-induced hypertension. In Part II, we took a first step in investigating the involvement of intestinal SIK1 activity in blood pressure regulation, focusing on the characterization of intestinal ion transport mechanisms that are potentially subject to SIK1 regulation. As described in our experimental study, the observed dexamethasone-induced modulation of basolateral K^+ currents, along with its effects on the regulation of Na^+-K^+-ATPase protein expression and activity, suggest a potential interplay between Na^+-K^+-ATPase and K_{ATP} channels in intestinal cells. As described in renal proximal tubule experimental models, this functional coupling between Na^+-K^+-ATPase activity and basolateral K^+ channel activity (pump-leak) is mediated by K_{ATP} channels and is of prime importance for epithelial ion transport – in fact, Na^+-K^+-ATPase activity regulation induces adjustments in basolateral K_{ATP} channel activity to the same extent as adjustments in basolateral K_{ATP} channel activity are required for Na^+-K^+-ATPase activity regulation[45].

Salt-inducible kinase 1 is known to affect the catalytic properties of the Na^+-K^+-ATPase units present at the plasma membrane by enhancing their activity in response to elevated intracellular sodium without interfering with NKA recruitment[6]. Since intestinal Na^+-K^+-ATPase activity dysregulation and abnormal sodium homeostasis also comprise key molecular mechanisms responsible for blood pressure rise under chronic high salt intake, we believe it is valuable to evaluate the role of SIK1 in intestinal sodium transport mechanisms, in particular the pump-leak coupling between Na^+-K^+-ATPase and K_{ATP} channels. Future investigations with cells transiently or stably lacking SIK1 using siRNA or shRNA and *in vivo* studies with *sik1*$^{-/-}$ mice are needed for better comprehension of the underlying physiologic mechanisms and to assess whether SIK1 blocking affects sodium balance and blood pressure through intestinal mechanisms.