Increased Arterial Blood Pressure and Vascular Remodeling in Mice Lacking Salt-Inducible Kinase 1 (SIK1)

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ABSTRACT

**Rationale:** In human genetic studies a single nucleotide polymorphism within the salt-inducible kinase 1 (SIK1) gene was associated with hypertension. Lower SIK1 activity in vascular smooth muscle cells (VSMCs) leads to decreased Na⁺,K⁺-ATPase activity, which associates with increased vascular tone. Also, SIK1 participates in a negative feedback mechanism on the transforming growth factor-β1 (TGFβ1) signaling and down-regulation of SIK1 induces the expression of extracellular matrix remodeling genes.

**Objective:** To evaluate whether reduced expression/activity of SIK1 alone or in combination with elevated salt intake could modify the structure and/or function of the vasculature, leading to higher blood pressure.

**Methods and Results:** SIK1 knockout (sik1⁻/⁻) and wild-type (sik1⁺/⁺) mice were challenged to a normal- or chronic high-salt intake (1% NaCl). Under normal-salt conditions, the sik1⁻/⁻ mice showed increased collagen deposition in the aorta but similar blood pressure compared to the sik1⁺/⁺ mice. During high-salt intake, the sik1⁺/⁺ mice exhibited an increase in SIK1 expression in the VSMCs layer of the aorta, whereas the sik1⁻/⁻ mice exhibited up-regulated TGFβ1 signaling and increased expression of endothelin-1 and genes involved in VSMC contraction, higher systolic blood pressure and signs of cardiac hypertrophy. In vitro knockdown of SIK1 induced up-regulation of collagen in aortic adventitial fibroblasts, and enhanced the expression of contractile markers and of endothelin-1 in VSMCs.

**Conclusions:** Vascular SIK1 activation might represent a novel mechanism involved in the prevention of high blood pressure development triggered by high-salt intake through the modulation of the contractile phenotype of VSMCs via TGFβ1-signaling inhibition.

**Keywords:**
SIK1, salt-induced hypertension, vascular remodeling, contractile smooth muscle cell phenotype, salt sensitivity hypertension, salt intake, vascular smooth muscle, endothelin-1

**Nonstandard Abbreviations and Acronyms:**
- SIK1: Salt-inducible kinase-1
- EN1: Endothelin-1
- AFs: Aortic adventitial fibroblasts
- VSMCs: Vascular smooth muscle cells
- Na⁺,K⁺-ATPase: Sodium-potassium adenosine triphosphatase
- ECM: Extracellular matrix
- MMPs: Matrix metalloproteinases
- NS: Normal-salt
- HS: High-salt
- Ser: Serine
- Gly: Glycine
- TGFβ1: Transforming growth factor β1
- LV: Left ventricle
- GFR: Glomerular filtration rate
- FE Na⁺: Fractional excretion of sodium
INTRODUCTION

Salt-inducible kinase (SIK) is a member of the AMP-activated protein kinase (AMPK) family of serine/treonine kinases. SIK1 was demonstrated to be elevated in the adrenal glands from animals under a high-salt diet. In this tissue, it inhibits the production of aldosterone by modulating cAMP response element-binding (CREB) activity directly or via transducer of regulated CREB (TORC) phosphorylation. In addition, SIK1 regulates active sodium transport in renal and lung epithelia by increasing Na⁺,K⁺-ATPase activity and mediates gene expression activation in cardiac myocytes upon increase in intracellular sodium.

SIK1 is also present in the vasculature and its activity in endothelial and vascular smooth muscle cells (VSMCs) appears to be relevant for controlling the vascular tone and arterial blood pressure by regulating Na⁺,K⁺-ATPase activity in VSMCs. A polymorphism in SIK1 gene resulting in one amino acid change (Gly→Ser) in the protein enhances the kinase activity and is associated with lower blood pressure and reduced left ventricle (LV) mass index in humans. Over-expression of the rare allele (SIK1-15Ser) in VSMCs leads to higher Na⁺,K⁺-ATPase activity. An increase in Na⁺,K⁺-ATPase activity is associated with a decrease in intracellular Ca²⁺ concentration which impairs contractility leading to reduced VSMCs tone; therefore this may indicate a role of vascular SIK1 activity on vascular tone relaxation that could in part explain the lower arterial blood pressure observed in human genetic studies.

Elevated arterial blood pressure can occur as a consequence of increased vascular stiffness attributed to both extracellular matrix (ECM) deposition/remodeling or enhanced contractility/stiffness of VSMCs. Transforming growth factor-β1 (TGFβ1) is a pleiotropic cytokine that mediates ECM remodeling processes within the vasculature in addition to promote VSMCs differentiation towards a contractile phenotype. SIK1 participates in a negative feedback mechanism on the TGFβ1 signaling pathway. We have reported that in epithelial cells the loss of SIK1 increased the expression of SNAI2 and TWIST1, known transcription factors involved in fibrosis and ECM remodeling.

Recently, brain SIK1 activity has been shown to be relevant for blood pressure regulation in rodents by regulating sympathetic activity. Higher hypothalamic activity of SIK1 was associated with reduced sympatho-excitatory activity and lower arterial blood pressure in rats after a high-salt diet and intracerebroventricular infusion of Na⁺.

Because SIK1 is present in the vasculature and lower SIK1 activity was associated with elevated blood pressure in humans and rodents, we hypothesize that the lack of SIK1 could trigger vascular remodeling processes leading to increased vascular stiffness and consequently to higher blood pressure. Furthermore, these events alone or in combination with other risk factors (high-salt intake) may contribute to the development of high blood pressure.
METHODS

**Generation of sik1−/− mice and experimental.**
The sik1−/− mice has been previously described.5 All animals were maintained under standard conditions of light (7 a.m., 7 p.m.) and temperature (22°C, 55% humidity), with free access to chow diet and tap water. At 10 weeks-old the sik1−/− and sik1+/+ mice remained on a chow diet but they were challenged to either normal-salt (NS, tap water) or high-salt (HS, 1% NaCl in tap water) intake for 14 weeks. The animals at 8 or 24 weeks-old were sacrificed and tissue samples were extracted for further analysis. The care and husbandry of animals were in accordance with European Directives no.86/609. Ethics committees at the Karolinska Institute, University of Porto, and CVMD - AstraZeneca Sweden approved all experimental protocols.

**Blood pressure measurements.**
Implantable telemetry was used for assessing blood pressure and heart rate. Mice were anesthetized by intraperitoneal injection (10 ml/kg) of ketamine (150 mg/kg), medetomidine (1 mg/kg) and butorfanol (1 mg/kg) in 0.9% saline. Mice were instrumented with radio-telemeters (TA11PA-C10, DSI, USA) as described elsewhere.21 Briefly, animals were placed on their backs on a heated pad and a 1-2 cm incision at the level of the ventral neck was performed. Salivary glands were gently separated and the right common carotid artery was carefully isolated. Telemetry transmitter was inserted into the carotid after cranial permanent ligation and temporary caudal occlusion. Catheter tip was positioned and secured in the aortic arch. The body of the device was slipped in a subcutaneous pocket and inserted through the same neck incision, on the left flank, as close as possible to the left hindlimb of the animal. Lastly, the skin was sutured with 6/0 skin suture. Post-operative care: wound closure was covered with 2% lidocaine cream and carprofen (5 mg/kg/day, subcutaneously) was administered for 3 days, twice daily. Animals were allowed to recover individually before experiments. After recovery (5 days after surgery), telemetry probes were magnetically turned on and blood pressure, heart rate and locomotor activity were monitored. Data were recorded for 40 seconds every 10 minutes for 72 h using Dataquest A.R.T. Acquisition and Analysis system 4.0 (DSI) (Online Figure I).

**Cardiac function and morphology.**
The left ventricle (LV) anterior and posterior wall thickness and LV systolic function was examined using transthoracic ultrasound technique.22, 23 Before ultrasound examination, the mice were anaesthetized using 4% isoflurane mixed to air, in a perforated chamber. When anesthetic induction was complete, the mice were placed in nose-cone for gas isoflurane (1%) anesthetics maintenance. The chest fur was removed. During the examination the mice were kept on a Physio Plate (Visualsonics, Canada) to keep normal body temperature and to monitor ECG and respiration. The ultrasound probe (MS400, 18–38 MHz) was mounted on a rail system (Visualsonics, Canada) and connected to an ultrasound biomicroscope (Vevo® 2100 System, Visualsonics, Canada). An LV long-axis parasternal B-mode in long-axis view was captured, followed by a 90° clockwise rotation of the ultrasound probe adjusted to the level just caudal to the mitral level to obtain short-axis B-MODE sequences and motion-mode (MMODE). Anterior and posterior wall thickness as well as end-diastolic LV dimension was measured using the American Society of Echocardiography leading edge. End-diastole was defined at the R-tag in the ECG signal. LV wall thickness was calculated as the average of the anterior and the inferior wall thickness. Fractional Shortening (FS) was calculated from MMODE measurements as follows: FS (%) = [(LVDd-LVDs)/LVDd]×100; (LVDd, LV end-diastolic diameter; LVDs, LV end-systolic diameter).

**Renal function.**
After the telemetry recording, mice were placed in metabolic cages (Tecniplast, Buguggiate, Italy) for a 24h urine collection. The urine samples were collected in vials that were subsequently stored at -80 °C until assayed. After completion of this protocol, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The animals were then sacrificed by exsanguination using cardiac puncture and the blood collected into tubes containing K3 EDTA for later determination of plasma biochemical parameters. All biochemical
assays were performed by Cobas Mira Plus analyzer (ABX Diagnostics for Cobas Mira, Switzerland). Aldosterone measurement in plasma samples was performed by radioimmuno assay (Diagnostic Products Corporation; Los Angeles, CA).

**Assay of norepinephrine.**
Blood was collected by cardiac puncture in pentobarbital-anesthetized animals. The assay of plasma norepinephrine was performed by HPLC with electrochemical detection, as previously described. 24

**Cell cultures and transfection.**
Human aortic adventitial fibroblasts (CC-7014) and human vascular smooth muscle cells (CC-2571) were obtained from Lonza (Lonza, Walkersville, USA) and maintained in SCGM™ or in SmGM™ (Lonza, Walkersville, USA), respectively. Transfections were performed using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturers’ instructions. The transfection efficiency, monitored by the expression of a fluorescent-labeled control siRNA by flow cytometry, was ~90% for both cells types. Cells (80,000) were plated one day before transfection on 6-well plates and transfected at 40% confluence for 24h, followed by incubation with or without 2.5 ng/ml of TGFβ1 (R&D Systems, Minneapolis, USA) for 24h. Scrambled- (sc-37007) and human SIK1-siRNA (sc-91428) were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA). Studies were performed between cell passages 3-6.

**Protein expression analysis.**
Tissue samples and cell cultures were lysed as described elsewhere.5 Protein samples (20-50 µg) were run on SDS-PAGE and Western Blot was performed. The membranes were probed with antibodies against: 1:500 COL1A1 (#SAB1402151, Sigma-Aldrich), 1:500 COL3A1 (sc-28888, Santa Cruz Biotechnology), 1:500 COL6A1 (sc-377143, Santa Cruz Biotechnology) and β-Actin or β-tubulin as loading controls (Sigma-Aldrich).

**Determination of mRNA expression.**
Aorta and heart tissue samples were directly incubated with RNAlater (Ambion, Austin, TX) and homogenized with a FastPrep using Lysing Matrix D tubes (MP Biomedicals, Germany). Reverse transcription and cDNA synthesis was performed as previously described.5 cDNA was amplified using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster city, CA). The relative amount of the mRNA of interest was normalized against to ribosomal protein, large, P0 (RPLP0) and TATA box binding protein (TBP) mRNAs using the comparative Ct-method.

**Immunohistochemistry.**
Samples from the abdominal aorta were frozen in Tissue-Tek Oct medium (Sakura Finetek USA Inc, Torrance, CA, USA) for histology analysis. Sections were fixed in acetone and stained with Mayer’s Haematoxylin (Histolab Products, Göteborg, Sweden) for measurement of lumen diameter and intima/media thickness at x20 or x40 magnification. Immunodetection was performed using a monoclonal antibody against SIK1 (1:2000) (#SAB1402668, Sigma-Aldrich), a polyclonal antibody against EN1 (1:3000) (sc-21625, Santa Cruz Biotechnology), a monoclonal antibody against TGFβ1 (40 ug/ml) (#MAB240, R&R Systems) and a polyclonal antibody against phospho-SMAD2/3 (1:50) (#8828, Cell Signaling) respectively, and counterstained with Mayer’s Haematoxylin as described previously.7 Picrosirius red staining was used for the assessment of collagen fibers in the vessels. Sections were stained for 1h in saturated picric acid containing 0.1% picrosirius red (Direct Red 80, Fluka, Buchs, Switzerland). This technique allowed for the discrimination of thick (red-orange staining) from thin (green staining) collagen fibers, which represent mature and immature collagen fibers. The total collagen content was measured using the Leica QWin software. All sections were analyzed under linear polarized light at x40 magnification.
Statistics.
Results are expressed as mean ± SEM or box plots (t-test or Mann-Whitney as appropriate) and plotted using GraphPad Prism software. P values less than 0.05 were considered statistically significant.

RESULTS

Loss of SIK1 triggers an increase in blood pressure upon a chronic high-salt intake in mice.

Blood pressure measurements and cardiac parameters were assessed on the sik1−/− and sik1+/+ mice challenged to either a NS or HS for 14 weeks. Body weight and heart rate (Figure 1A-B) were similar among all experimental groups. No differences in blood pressure levels were observed between the sik1−/− and sik1+/+ mice under NS. However, upon a chronic HS intake systolic blood pressure was significantly greater in the sik1−/− mice (135.0±4.3 mmHg) when compared to the sik1+/+ mice (122.1±1.9 mmHg), whereas diastolic and mean arterial blood (Figure 1C-E) pressure were similar in both groups. Systolic blood pressure relates more closely to vascular resistance; thus, this result might reflect an increased arterial stiffness in the sik1−/− mice.

The ultrasound examination of the hearts of these mice revealed that under HS the sik1−/− mice have an increased LV wall thickness and a decreased LV diameter (Online Figure IIA and Figure 2A-B) when compared to the sik1+/+ mice. In addition, LV+septum weight/body weight ratio was significantly increased in the sik1−/− mice, while no changes were observed in right ventricle weight/body weight or heart weight/body weight (Figure 2C-E), further confirming the presence of a hypertrophic heart in the sik1−/− mice. No changes were observed in the fractional shortening in neither diet nor genotype (Online Figure IIB). At the molecular level, the mRNA expression of the transcription factor MEF2C, as well as structural components of the cardiomyocytes like skeletal actin and β-myosin heavy chain (Online Figure IIC-E) were significantly elevated in the sik1−/− versus the sik1+/+ mouse group only under HS intake.

These results suggest that the lack of SIK1 renders the mice salt-sensitive and demonstrate the relevance of SIK1 in blood pressure regulation upon a HS intake.

Increased natriuresis but preserved sympathetic activity in the sik1−/− mice.

Increases in blood pressure can arise from an enhanced sympathetic drive or from an abnormal secretion of numerous hormones and peptides that participates in water and sodium homeostasis as a consequence of a deteriorated renal function. Renal function and sympathetic activity (plasma norepinephrine) were evaluated in the sik1−/− and sik1+/+ mice and the results are summarized in Table 1 and Online Table I, respectively. Water intake and urinary volume were similar between both genotypes under both NS and HS intakes. During HS intake water consumption increased significantly (~50%) in both groups and this was accompanied by an increase in urinary volume. Plasma creatinine and creatinine clearance showed no differences between both genotypes under neither NS nor HS intake, indicating a preserved glomerular filtration rate (GFR) in the knockout mice. Urinary protein concentration as well as fractional excretion of sodium (FENa+) and potassium (FEK+) was similar for both genotypes, indicating no signs of renal damage. The daily urinary excretion of Na+, K+ and Cl− was increased in the knockout mice under both treatments, despite this, their plasma electrolytes concentrations (Na+, K+, Cl−) were normal. Plasma aldosterone levels were similar between the sik1−/− and sik1+/+ mice under both NS and HS intake and decreased significantly during HS to a similar extent in both groups. Likewise, no differences were observed in plasmatic norepinephrine levels and heart rate between the sik1−/− and sik1+/+ mice under both NS and HS intake (Online Table I and Figure 1B). At the molecular level, the mRNA expression of SIK1 and SIK2 in kidney samples from the sik1+/+ mice was significantly reduced upon HS intake, whereas...
no variations in the expression of SIK2 and SIK3 isoforms where observed in the sik1⁻/⁻ mice (Online Figure III). Apart from an enhanced urinary excretion of electrolytes, the loss of SIK1 does not significantly affect the normal function of the kidneys or sympathetic activity suggesting that blood pressure variations are most probably due to an extra-renal action of SIK1.

Loss of SIK1 induces vascular remodeling through the modulation of adventitial fibroblasts and smooth muscle cells phenotype.

Histological analysis of abdominal aorta specimens from young mice (8 weeks-old) showed no changes in vessel diameter but a significant increase in intima/media thickness (Figure 3A-B) in those from the sik1⁻/⁻ mice. In addition, the loss of SIK1 resulted in a significant increase of immature collagen fibers (newly synthesized collagen) compared to the wild-type mice (Figure 3C). In the aortas of the sik1⁻/⁻ mice there was a significant increase in the expression of SNAIL1 and TWIST1 mRNAs, together with a parallel up-regulation of several collagen isoforms at the mRNA (Figure 3D, left) and at the protein level (Figure 3E, right). COL6A1 gene was not detected at the transcriptional level due to its low turnover. Furthermore, MMP-9, N- and VE-cadherin mRNAs were also up-regulated by the loss of SIK1 (Figure 3D, left). Thus, lacking SIK1 drives an advanced tissue remodeling process within the vasculature characterized by an increased fibrosis.

Vascular collagen deposition was further evaluated in adult mice (24 weeks-old) after NS or chronic HS intake. Abdominal aorta diameter was similar among all experimental groups (Figure 4A). Under NS, collagen deposition was similar as observed in aortas from young mice; there was a significant increase in immature collagen fibers (Figure 4B) whereas no changes in mature collagen fibers content (Figure 4C) in the sik1⁻/⁻ mice compared to the wild-type mice. This result is consistent with the gene expression data, where the transcription of collagen isoforms was increased in aortas from the sik1⁻/⁻ mice (Figure 4D-F). Interestingly, under HS intake the situation changes significantly. The loss of SIK1 in combination with a HS intake did not affect immature collagen fibers deposition (Figure 4B), meanwhile it decreased mature collagen fibers content (Figure 4C) when compared to wild-type mice aortas. This can be explained by the inhibition of collagen transcription in the knockout mice (Figure 4D-F), contrary to the observed under NS. Also, the decrease in mature collagen fibers indicates an enhanced collagen degradation process, which could be due to an increased activity of MMPs induced by HS. Furthermore, the loss of SIK1 significantly increased SNAI1, and VE-cadherin expression under both NS and HS intake (Online Figure IV). Of note, the chronic HS intake did not modify the deposition of collagen in the aortas of the wild-type mice (mature collagen fibers, AU=3.72±1.42 vs. 3.72±0.78; immature collagen fibers, AU=2.73±0.95 vs. 3.07±0.59 vs. WT-HS diet) (Figure 4B-C).

Next, transient knockdown of SIK1 (using siRNAs) in the presence of TGFβ1 in primary cell cultures of human aortic adventitial fibroblasts (AFs) significantly enhanced the expression of collagen isoforms both at the mRNA and at the protein level (Figure 5A), consistent with the notion of SIK1 being a negative regulator of the TGFβ1 signaling. On the contrary, SIK1 knockdown in primary cell cultures of human VSMCs did not alter collagen expression (Figure 5B) but it did affect the expression of several genes involved in the acquisition of a differentiated contractile phenotype. The expression of myocardin, smoothelin and N-cadherin were highly up-regulated (~3-4 fold increase) when compared to control siRNA-transfected cells (Figure 5C). VSMCs are also an important source of the vasoconstrictor factor endothelin-1 (EN1). The mRNA expression of EN1, EN1 receptor type A (ENRA) - which mediates vasoconstriction via increasing intracellular calcium signaling - and EN converting enzyme 1 (ECE1) - one of the enzymes responsible for processing EN1 into the mature peptide - was significantly elevated in SIK1 knockdown cells. Moreover, the mRNA expression of genes involved in cell cycle progression like forkhead box protein M1 (FOXM1) and polo-like kinase 1 (PLK1) was significantly diminished (Figure 5C). TGFβ1 incubation in AFs and VSMCs in culture significantly induced the SIK1 isoform without major changes in SIK2 and SIK3 (Online Figure VA-B). These results show that the loss of SIK1 apart from
triggering collagen deposition by an increased activity of AFs, it also promotes the phenotypic switch of VSMCs from synthetic-proliferative to contractile.

We next evaluated whether SIK1 can modulate the expression of these genes in vivo. The mRNA levels of these genes in NS were similar in both genotypes, except for N-cadherin that was up-regulated in the sik1-/- mice. α-smooth muscle actin was up-regulated by HS in both groups of mice, independently of SIK1 expression (Figure 6A). During HS intake there was a significant up-regulation (~50%) of myocardin, smoothelin and N-cadherin in the sik1-/- mice (Figure 6B-D). Immunohistochemistry analysis of aorta sections revealed increased EN1 expression throughout the media layer in the sik1-/- mice compared to the wild-type mice under HS, whereas high expression was observed in the endothelial cell layer in both mice (Figure 6E). SIK1 was highly expressed in the endothelial and adventitial layers and to a lesser extent in the media layer (sub-endothelium mainly) of the abdominal aorta of wild-type mice on NS (Figure 7A). In this tissue upon chronic HS intake, SIK1 expression increased specifically throughout the media layer but more markedly in the sub-endothelium and remained high in endothelial and adventitial layers. SIK1 mRNA levels increased during HS while no changes were observed for the other SIK isoforms at any condition (Figure 7B and Online Figure VC). Additionally, during HS intake TGFβ1 mRNA and protein levels were higher in aorta sections from the sik1-/- mice compared to the wild-type mice, and similarly an increase of phospho-SMAD2/3 staining was observed (Figure 7C-E).

Together these results suggest that the loss of SIK1 drives a tissue remodeling process within the vasculature as a consequence of a dysregulated fibrotic phenotype of AFs, as well as an increased contractile phenotype of VSMCs due to enhanced EN1 and TGFβ1 signaling.

**DISCUSSION**

Many pathophysiologic and environmental factors have been implicated in the genesis of hypertension.27 Among these, HS intake contributes to the elevation of arterial blood pressure in humans and animal models.11, 27-31 In human genetic studies, the identification of a single nucleotide polymorphism in the coding region of SIK1 gene that was associated with hypertension provided us an insight on the potential role of SIK1 in the development of hypertension.7 In the present study we show that the loss of SIK1 contributes to the development of high blood pressure triggered by a chronic HS intake in mice. The negative impact on blood pressure was associated with alterations of the vasculature, e.g. dysregulated collagen synthesis and increased contractile phenotype of VSMCs as well as increased EN1 expression. SIK1 was initially discovered in adrenal glands of rats fed with a HS diet and it has been shown to act as a mediator during the cellular adaptation to variations in intracellular sodium in a variety of cell types.4, 6 In agreement with previous literature, we observed that the regulatory action of SIK1 on blood pressure levels is exerted specifically during abnormal salt intake. Furthermore, our results showed that within the vasculature SIK1 was induced in the VSMCs layer of the wild-type mice aorta in response to the chronic HS intake.

As mentioned previously, hypertension is the result of complex interactions between genetic and environmental factors. Despite this, it is believed that renal mechanisms play a primary role in blood pressure increase through impairment of sodium and water handling.25 SIK1 participates in sodium reabsorption in the proximal convoluted tubule via modulating Na⁺,K⁺-ATPase activity and is involved in the establishment of a polarized epithelium which is essential for the vectorial transport of ions and solutes via controlling E-cadherin availability in the plasma membrane.4, 5 The results presented here showed no differences in renal function between the sik1-/- and sik1+/+ mice after 14 weeks of NS or HS intake (GFR, FENa⁺, plasma electrolytes and aldosterone levels were similar between both genotypes), except for urinary Na⁺ excretion. The knockout mice showed increased natriuresis during HS intake, effect that was also
observed during NS where blood pressure levels were normal. This phenotype is consistent with decreased activity of the \( \text{Na}^+, \text{K}^- \)-ATPase and also possibly with down-regulation of E-cadherin in the renal epithelium as we previously showed. One possible explanation for the increase in urinary \( \text{Na}^+ \) and \( \text{K}^- \) in the \( \text{sik1}^{-/-} \) mice without evidence of severe sodium depletion, as indicated by normal plasma \( \text{Na}^+ \) and \( \text{K}^- \) levels, is that of a possible intestinal absorptive compensatory mechanism to keep electrolyte homeostasis.\(^{32-35}\) Although we cannot exclude the possibility that renal function is affected in the \( \text{sik1}^{-/-} \) mice during the first days/weeks of HS intake, it is unlikely that the loss of renal SIK1 is responsible for the mild hypertension given that increased urinary \( \text{Na}^+ \) excretion, and not the opposite, is observed in these mice. Aldosterone levels did not differ in the \( \text{sik1}^{-/-} \) mice, therefore it is plausible to think that in the adrenal gland the other SIK isoforms could display compensatory mechanisms in order to maintain TORC phosphorylation levels and indeed in vitro studies in adrenocortical cells showed that SIK2 and SIK3 also phosphorylate this co-factor.\(^{3,36}\)

It has been described that HS intake increases endothelial production of TGF\( \beta \)1 which can act in a paracrine fashion on the vessel wall participating in VSMCs differentiation towards a contractile phenotype.\(^ {18,37-39}\) Here, we showed that in the vasculature of the \( \text{sik1}^{-/-} \) mice the HS intake increased TGF\( \beta \)1 expression and downstream phospho-SMAD2/3 signaling compared to the wild-type mice, which suggests that the contractile phenotype of VSMCs should be favored in the \( \text{sik1}^{-/-} \) mice. Indeed, we found increased expression of contractile genes and of EN1 in the knockout compared to the wild-type mice aortas, only during HS intake. The role of SIK1 in VSMCs differentiation was further confirmed in vitro where SIK1 knockdown in VSMCs resulted in an enhanced expression of contractile genes as well as EN1, ENRA and ECE1, while the expression of proliferative genes was diminished. On the other hand, in the aorta of the \( \text{sik1}^{-/-} \) mice the HS intake caused an increase of SIK1 expression in the VSMCs layer which in turn negatively regulates TGF\( \beta \)1 signaling, therefore inhibiting VSMCs differentiation. Thus, these results suggest that the loss (or reduced activity) of SIK1 in combination with HS intake predispose to an active contractile phenotype of VSMCs, setting to a higher vascular tone, and thereby contributing to higher blood pressure. Therefore, SIK1 activation in the vasculature may function as a protective mechanism to maintain a relaxed VSMCs tone and ultimately a lower blood pressure.

It is well recognized that vascular changes are associated with cardiovascular diseases, and hypertension.\(^ {10-12,40}\) Systolic blood pressure in particular increases with age and is related to arterial stiffness of large conduit arteries.\(^ {41,42}\) We observed an increased collagen deposition in the aorta of young and adult NS-fed knockout mice compared to wild-type mice. Similarly, SIK1 knockdown in AFs showed an enhanced expression of collagen isoforms compared to control cells. The increase in collagen deposition observed in the knockout mice under NS did not affect blood pressure levels, suggesting that collagen deposition \textit{per se} is not sufficient to trigger high blood pressure, at least at the time points tested. We cannot exclude the possibility that these mice will develop hypertension in later stages of life due to the increased vascular fibrosis. Interestingly, upon HS intake the knockout mice, which developed high blood pressure, showed significantly less mature collagen fibers in the aortic wall than the wild-type mice. Although contradictory, these results show that SIK1 can modulate collagen synthesis and degradation in a cell- and context-specific fashion. Whether a reduction in collagen fibers in the vasculature may favor an increase in blood pressure upon HS intake deserves further studies, but we can speculate that modifications of the vascular ECM composition by modifying the availability or activity of salt-inducible vasoactive compounds could affect the vascular tone.

SIK1 is also present in the brain and it has been proposed that dysregulation of the SIK1- Na\(^+\),K\(^-\)-ATPase network in neurons contributes to the salt-induced hypertension through enhanced angiotensinergic sympathetic hyperactivity by increasing intracellular \( \text{Ca}^{2+} \).\(^ {20,43}\) Our results showed no differences in sympathetic activity between the knockout and wild-type mice as plasma norepinephrine levels remained unaffected. However, it should be noted that plasma norepinephrine values provide only limited assessment of sympathetic control of blood pressure in conscious animals.

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In addition, heart rate and locomotor activity were similar between both genotypes at any given condition. The reason for these differences could be due to differences in methodological approaches used in each study.

In this study we demonstrate the importance of vascular SIK1 activity for blood pressure regulation under chronic HS intake; however we cannot exclude the possibility that SIK1 present in other tissues could also be of relevance by affecting the release, availability and/or the activity of unknown salt-inducible mediators. Ideally, a vascular specific SIK1 knockout mouse model would provide more accurate information regarding the relative contribution of vascular vs. non-vascular SIK1 activity.

In conclusion, this study confirms and extends our previous observations in human genetic studies and highlights the relevance of SIK1 in blood pressure regulation in vivo. Vascular SIK1 activity is necessary to prevent the development of high blood pressure triggered by a chronic HS intake. Therefore, efforts aimed at increasing SIK1 expression or activity could become an interesting strategy for the treatment/prevention of salt-sensitive hypertension as well as for vascular fibrosis-related disorders.

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DISCLOSURES
None.
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FIGURE LEGENDS

Figure 1. Blood pressure in the sik1+/+ (WT) and sik1−/− (KO) mice under normal (NS) or high-salt (HS) intake. Body weight (A), Heart rate (HR) (B) and radio-telemetry recordings of (C) Systolic blood pressure (SBP), (D) Diastolic blood pressure (DBP) and (E) Mean arterial blood pressure (MAP). Mean±SEM; n=6-7; ns: not significant.

Figure 2. Cardiac parameters in the sik1+/+ (WT) and sik1−/− (KO) mice under normal (NS) or high-salt (HS) intake. Quantitative analysis of left ventricular (A) wall thickness (LVWT) and (B) diameter (LVD) performed by transthoracic ultrasound. (C) LV+septum weight/body weight (LV+SW/BW), (D) right ventricle weight/body weight (RVW/BW) and (E) heart weight/body weight (HW/BW) ratios. Mean±SEM or box plots; n=6-7; ns: not significant.

Figure 3. Histochemistry and gene expression analysis of aortas from young sik1+/+ (WT) and sik1−/− (KO) mice. Representative image (left panels) and the corresponding quantifications (right panels) of abdominal aorta sections from 8-weeks-old mice stained with (A-B) haematoxilin showing (A) lumen diameter (x20) and (B) intima/media thickness (x40); and (C) picrosirius red coupled to polarized light microscopy (x40) showing immature collagen fibers (thin fibers = green) and mature collagen fibers (thick fibers = red-orange). (D) mRNA (left panel) and protein (right panel) expression in the sik1+/+ and sik1−/− mice aortas. Representative Western blots are shown. OD=optical density, was calculated normalizing the specific signal against β-tubulin signal. Mean±SEM or box plots; n=6-7; ns: not significant. *p<0.05; **p<0.01.

Figure 4. Histochemistry and gene expression analysis of aortas from the sik1+/+ (WT) and sik1−/− (KO) mice under normal (NS) or high-salt (HS) intake. Quantitative measurement of abdominal aorta (A) lumen diameter; (B) immature and (C) mature collagen fibers from picrosirius red staining. (D-F) mRNA expression in the sik1+/+ and sik1−/− mice aortas. Mean±SEM or box plots; n=6-7; ns: not significant.

Figure 5. Gene expression analysis in human aortic adventitial fibroblasts (AFs) and in human vascular smooth muscle cells (VSMCs). Primary cultures of AFs (A) and VSMCs (B-C) were transfected with either SIK1- (si-SIK1) or scrambled- (si-SCR) siRNAs for 24h and TGF1-stimulated for additional 24h. (A) mRNA and protein levels in AFs. Representative Western blots are shown. OD=optical density, was calculated normalizing the specific signal against -actin signal. (B) mRNA of fibrotic and (C) contractile genes in VSMCs. Mean±SEM; n=3. *p<0.05; **p<0.01; ***p<0.001 vs. si-SCR cells.

Figure 6. Gene expression analysis in aortas from the sik1+/+ (WT) and sik1−/− (KO) mice under normal (NS) or high-salt (HS) intake. (A-D) mRNA levels of contractile genes in the sik1+/+ and sik1−/− mice under NS or HS. Mean±SEM; n=6-7. *p<0.05, vs. WT-NS aortas; b*p<0.05, vs. KO-NS aortas. (E) Representative photomicrographs of immunohistochemical staining performed with an antibody against EN1 in abdominal aorta tissue sections from sik1+/+ and sik1−/− mice under HS (x40). Sections were counterstained with Mayer’s Haematoxylin.

Figure 7. Immunolocalization of SIK1 and TGFβ1 in aortas from the sik1+/+ (WT) and sik1−/− (KO) mice under normal (NS) or high-salt (HS) intake. (A) Representative photomicrographs of immunohistochemical staining performed with an antibody against SIK1 in abdominal aorta tissue sections from sik1+/+ mice. (B) mRNA levels of SIK1 in abdominal aorta of sik1+/+ mice under NS or HS. (C) Representative photomicrographs of immunohistochemical staining performed with an antibody against TGFβ1 in abdominal aorta tissue sections from sik1+/+ and sik1−/− mice under HS. (D) mRNA levels of TGFβ1 in abdominal aorta of sik1+/+ and sik1−/− mice under HS. *p<0.05, vs. WT-NS; b*p<0.05, vs. WT-HS. (E) Quantification of immunohistochemical detection of phospho-SMAD2/3 positive nuclei in intima (of...
the whole tissue section) and media (of a random area of the tissue section) layers of abdominal aorta from sik1+/+ and sik1−/− mice under HS. *p<0.05; **p<0.01 vs. WT-HS. Whole aortas (x20) and specific areas (x40) are shown. All sections were counterstained with Mayer’s Haematoxylin. Arrows indicate positive SIK1 staining in the intima and adventitia layers, whereas asterisk in the media layer. Mean±SEM; n=6-7.
Table 1. Renal function and plasma electrolytes in the sik1+/+ and sik1−/− mice under normal (tap water) or high-salt (1% saline) intake.

<table>
<thead>
<tr>
<th></th>
<th>sik1+/+</th>
<th>sik1−/−</th>
<th>sik1+/+</th>
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<tbody>
<tr>
<td></td>
<td>tap water</td>
<td>tap water</td>
<td>1% NaCl</td>
<td>1% NaCl</td>
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<tr>
<td>Body weight (g)</td>
<td>28.2±0.9</td>
<td>27.4±1.3</td>
<td>29.3±1.6</td>
<td>27.4±1.2</td>
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<td>Kidney weight (weight/body weight)</td>
<td>12.2±0.8</td>
<td>12.9±1.1</td>
<td>13.0±0.8</td>
<td>13.8±0.6</td>
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<td>Water intake (ml/24h)</td>
<td>2.58±0.31</td>
<td>3.20±0.61</td>
<td>3.98±0.28 *</td>
<td>4.49±0.63 *</td>
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<tr>
<td>Urinary volume (ml/24h)</td>
<td>1.00±0.21</td>
<td>0.71±0.07</td>
<td>1.40±0.20</td>
<td>0.94±0.13</td>
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<tr>
<td>Urinary creatinine (mg/24h)</td>
<td>0.71±0.06</td>
<td>0.56±0.05</td>
<td>0.70±0.07</td>
<td>0.57±0.04</td>
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<tr>
<td>Urinary urea (mg/24h)</td>
<td>118.1±16.3</td>
<td>103.8±7.9</td>
<td>129.9±14.1</td>
<td>118.7±9.4</td>
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<tr>
<td>Urinary protein (mg/24h)</td>
<td>6.0±1.0</td>
<td>4.1±1.0</td>
<td>5.9±1.4</td>
<td>4.1±0.9</td>
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<td>Urinary Na⁺ (μmol/24h)</td>
<td>38.3±8.2</td>
<td>93.9±21.4 *</td>
<td>231.3±53.5</td>
<td>500.0±97.2 §</td>
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<td>Urinary K⁺ (μmol/24h)</td>
<td>437.6±98.7</td>
<td>1054.1±222.0 *</td>
<td>294.1±66.5</td>
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<tr>
<td>Urinary Cl⁻ (μmol/24h)</td>
<td>261.3±53.5</td>
<td>596.8±120.3 *</td>
<td>402.8±84.5</td>
<td>912.7±233.1</td>
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<tr>
<td>FE Na⁺</td>
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<td>0.23±0.06</td>
<td>1.66±0.21</td>
<td>1.81±0.36</td>
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<td>FE K⁺</td>
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<td>57.9±5.9</td>
<td>50.2±4.5</td>
<td>54.7±4.7</td>
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<tr>
<td>Urinary sodium/potassium ratio</td>
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<td>0.094±0.019</td>
<td>0.776±0.054</td>
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<tr>
<td>Creatinine clearance (ml/min)</td>
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<td>Plasma creatinine (mg/dl)</td>
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<td>0.4±0.0</td>
<td>0.4±0.0</td>
<td>0.4±0.0</td>
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<tr>
<td>Plasma Na⁺ (mmol/l)</td>
<td>143.1±6.7</td>
<td>147.2±5.4</td>
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<td>Plasma K⁺ (mmol/l)</td>
<td>5.8±0.4</td>
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<td>Plasma Cl⁻ (mmol/l)</td>
<td>115.7±2.9</td>
<td>113.4±2.0</td>
<td>110.7±1.4</td>
<td>109.0±1.7</td>
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<tr>
<td>Plasma aldosterone (ng/dl)</td>
<td>16.9±4.3</td>
<td>24.7±6.0</td>
<td>4.6±1.0 *</td>
<td>4.5±1.2 *</td>
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</tbody>
</table>

FE = fractional excretion. Values are expressed as mean±SEM. * p<0.05 vs. sik1+/+ - tap water; § p<0.05 vs. sik1+/+ - 1% NaCl.

Renal function studies and plasma electrolytes on the sik1+/+ and sik1−/− mice under normal (tap water) or high-salt (1% saline) intake. Mice were placed in metabolic cages for 24h and urine and blood samples were collected. Mean±SEM; n=6-7.
Novelty and Significance

What Is Known?

- Salt-inducible kinase 1 (SIK1) is a serine/treonine kinase that is involved in the cellular adaptation to changes in intracellular sodium concentration by genomic and non-genomic effects.
- SIK1 controls expression/activity of transcription factors and increases the Na⁺, K⁺-ATPase activity.
- In addition, SIK1 negatively regulates transforming growth factor-β1 (TGFβ1) signaling pathway by facilitating TGFβ1 type I receptor turnover.
- A single nucleotide polymorphism within the SIK1 gene that results in increased kinase activity of the encoded protein is associated with lower blood pressure and reduced left ventricle mass index in humans.
- Increased arterial stiffness due to extracellular matrix deposition are enhance contractility of vascular smooth muscle cells (VSMCs), processes that are regulated by TGFβ1, play important roles in development of hypertension in humans and in animal models of hypertension.

What New Information Does This Article Contribute?

- Activation of vascular SIK1 maintains a dedifferentiated phenotype of VSMCs and is necessary for controlling blood pressure upon a high-salt intake.
- Down-regulation of SIK1 induces VSMCs differentiation towards a contractile phenotype through transcriptional up-regulation of contractile genes and endothelin-1 and inhibition of genes involved in cell cycle progression. The latter effect depends on enhanced TGFβ1 expression/signaling in the vasculature.
- SIK1 controls collagen deposition within the vessel walls by modulating adventitial fibroblasts activity.

Despite advances in treatment strategies, hypertension remains one of the major risk factors for cardiovascular diseases. Therefore, there is a growing interest to develop novel therapies for the prevention and treatment of hypertension. Previous studies proposed a role of SIK1 in the development of hypertension in humans however the precise mechanism is not fully elucidated. In this study we analyzed the cellular and physiological SIK1-dependent mechanisms involved in blood pressure regulation. We found that up-regulation of SIK1 in the vasculature is required for controlling blood pressure upon high-salt intake. Studies using SIK1 knockout mice challenged to chronic high-salt intake showed that the increase in blood pressure levels was associated with increased TGFβ1 expression and enhanced contractile phenotype of VSMCs within the aorta. On contrary, the salt-dependent increase in SIK1 expression in aortic VSMCs protected the wild-type mice against developing high blood pressure. In vitro studies demonstrated that the loss of SIK1 is required for the acquisition of a contractile phenotype of VSMCs in the presence of TGFβ1. These findings increase the knowledge of salt-dependent mechanisms leading to hypertension and suggest that therapeutic strategies targeting vascular SIK1 could be beneficial for the treatment of hypertension and other vascular fibrosis-related diseases.
Figure 4
Increased Arterial Blood Pressure and Vascular Remodeling in Mice Lacking Salt-Inducible Kinase 1 (SIK1)

Alejandro M Bertorello, Nuno M Pires, Bruno Igreja, Maria J Pinho, Emina Vorkapic, Dick Wågsäter, Johannes Wikström, Margareta Behrendt, Anders Hamsten, Per Eriksson, Patrício Soares-da-Silva and Laura Brion

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SUPPLEMENTAL MATERIAL

Increased arterial blood pressure and vascular remodeling in mice lacking Salt-Inducible kinase 1 (SIK1)


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†This study was initiated by Dr. Alejandro M. Bertorello. Unfortunately he passed away 23rd January 2013. All coauthors have agreed on the submission of the manuscript on his behalf.

Correspondence: Laura Brion, Karolinska University Hospital-Solna, CMM L8:03, 171 76 Stockholm, Sweden. Tel: +46 8 517 73201, E-mail: laura.brion@ki.se

Running title: SIK1, hypertension and vascular remodeling.
Online Figure I: Typical 24h circadian rhythms blood pressure and heart rate in the sik1+/+ (WT) and sik1−/− (KO) mice under normal (NS) or high-salt (HS) intake. Radio-telemetry recordings of (A) systolic blood pressure (SBP), (B) diastolic blood pressure (DBP), (C) mean arterial pressure (MAP) and (D) heart rate (HR) for 3 consecutive days following implantation of the telemetry device. Plots represent mean values over each 4h in a 12:12h light-dark cycle. Gray segments indicate the dark period (7p.m. to 7a.m.). Mean±SEM; n=6-7.
Online Figure II: Cardiac parameters and gene expression analysis in the sik1+/+ (WT) and sik1-/− (KO) mice under normal (NS) or high-salt (HS) intake. (A) Representative ultrasound images of the hearts indicating left ventricular anterior and posterior wall thickness and left ventricular end diastolic diameter. (B) Fractional shortening (FS). (C-D) mRNA expression of Myocyte-specific Enhancer Factor 2C (MEF2C), skeletal actin (ACTA1) and β-myosin heavy chain (MHY7) in the sik1+/+ and sik1-/− mice hearts. Mean±SEM; n=6-7.
Online Figure III: Gene expression analysis in the kidney of the sik1^{+/+} (WT) and sik1^{-/-} (KO) mice under normal (NS) or high-salt (HS) intake. The mRNA expression of all SIK isoforms in the sik1^{+/+} and sik1^{-/-} mice kidney. Mean±SEM; n=6-7. *p<0.05, vs. WT-NS.

Online Figure IV: Gene expression analysis in the aorta of the sik1^{+/+} (WT) and sik1^{-/-} (KO) mice under normal (NS) or high-salt (HS) intake. (A-D) mRNA expression in the sik1^{+/+} and sik1^{-/-} mice aortas. Mean±SEM; n=6-7.
Online Figure V: Gene expression analysis in human aortic adventitial fibroblasts (AFs), human vascular smooth muscle cells (VSMCs) and in the aorta of the sik1+/+(WT) and sik1−/−(KO) mice under normal (NS) or high-salt (HS) intake. Primary cultures of AFs (A) and VSMCs (B) were transfected with either SIK1- (si-SIK1) or scrambled- (si-SCR) siRNAs for 24h in the presence or absence of TGFβ1 for additional 24h and mRNA levels of all SIK isoforms were evaluated. (C) mRNA levels of SIK2 and SIK3 in the sik1+/+ and sik1−/− mice aortas upon NS or HS intake. Mean±SEM; n=6-7. a p<0.05, vs. si-scr-vehicle; b p<0.05, vs. si-scr-TGFβ1.

Online Table I. Plasma norepinephrine levels in the sik1+/+ and sik1−/− mice

<table>
<thead>
<tr>
<th>sik1+/+</th>
<th>sik1−/−</th>
<th>sik1+/+</th>
<th>sik1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>tap water</td>
<td>tap water</td>
<td>1% NaCl</td>
<td>1% NaCl</td>
</tr>
<tr>
<td>Norepinephrine (pmol/ml)</td>
<td>6.99±0.51</td>
<td>8.09±0.68</td>
<td>6.58±0.55</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM.

Online Table I: Sympathetic activity in the sik1+/+ and sik1−/− mice under normal (tap water) or high-salt (1% saline) intake. Blood samples were collected and plasma norepinephrine quantification was performed by HPLC with electrochemical detection. Mean±SEM; n=6-7.