Possible role of brain salt-inducible kinase 1 in responses to central sodium in Dahl rats

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Huang BS, White RA, Leenen FH. Possible role of brain salt-inducible kinase 1 in responses to central sodium in Dahl rats. Am J Physiol Regul Integr Comp Physiol 303: R236–R245, 2012. First published May 30, 2012; doi:10.1152/ajpregu.00381.2011.—In Dahl salt-sensitive (S) rats, Na+ entry into the cerebrospinal fluid (CSF) and sympathoexcitatory and pressor responses to CSF Na+ are enhanced. Salt-inducible kinase 1 (SIK1) increases Na+/K+-ATPase activity in kidney cells. We tested the possible role of SIK1 in regulation of CSF [Na+] and responses to Na+ in the brain. SIK1 protein and activity were lower in hypothalamic tissue of Dahl S (SS/Mcw) compared with salt-resistant SS.BN13 rats. Intracerebroventricular infusion of the protein kinase inhibitor staurosporine at 25 ng/day, to inhibit SIK1 further increased mean arterial pressure (MAP) and HR but did not affect the increase in CSF [Na+] or hypothalamic aldosterone in Dahl S on a high-salt diet. Intracerebroventricular infusion of Na+ rich artificial CSF caused significantly larger increases in renal sympathetic nerve activity, MAP, and HR in Dahl S vs. SS.BN13 or Wistar rats on a normal-salt diet. Intracerebroventricular injection of 5 ng staurosporine enhanced these responses, but the enhancement in Dahl S rats was only one-third that in SS.BN13 and Wistar rats. Staurosporine had no effect in Dahl S vs. SS.BN13 or Wistar rats on a normal-salt diet. Intracerebroventricular infusion of Na+ rich artificial CSF caused significantly larger increases in renal sympathetic nerve activity, MAP, and HR in Dahl S vs. SS.BN13 or Wistar rats on a normal-salt diet. Intracerebroventricular injection of 5 ng staurosporine enhanced these responses, but the enhancement in Dahl S rats was only one-third that in SS.BN13 and Wistar rats. Staurosporine had no effect on MAP and HR responses to intracerebroventricular ANG II or carbachol, whereas the specific protein kinase C inhibitor GF109203X inhibited pressor responses to intracerebroventricular Na+ rich artificial CSF or ANG II. These results suggest that the SIK1-Na+/K+-ATPase network in neurons acts to attenuate sympathoexcitatory and pressor responses to increases in brain [Na+]. The lower hypothalamic SIK1 activity and smaller effect of staurosporine in Dahl S rats suggest that impaired activation of neuronal SIK1 by Na+ may contribute to their enhanced central responses to sodium.

Salt-induced hypertension; renal sympathetic nerve activity

IN DAHL SALT-SENSITIVE (S) RATS, BOTH ENHANCED Na+ ENTRY FROM THE BLOOD INTO THE CEREBROSPINAL FLUID (CSF) AND ENHANCED NEURONAL EXCITATORY RESPONSES TO Na+ CONTRIBUTE TO SALT-INDUCED HYPERTEN SIVITY AND HYPERTENSION (17, 19). IN DAHL S RATS ON A HIGH-SALT DIET, ENHANCED Na+ TRANSPORT, IN PART, VIA Na+/K+-ATPASE IN THE CHOROID PLEXUS (1, 17), ENHANCES Na+ ENTRY INTO THE CSF AND CSF [Na+]. HIGH SALT INTAKE IN DAHL S RATS OR CHRONIC INTRACEREBROVENTRICULAR INFUSION OF Na+-RICH ARTIFICIAL CSF (aCSF) IN WISTAR RATS APPEARS TO INCREASE LOCAL PRODUCTION OF ALDOSTERONE IN THE HYPOTHALAMUS (14, 20) AND THEREBY INCREASES RELEASE OF ENDOGENOUS OUAHIN-LIKE FACTOR (EO) (14, 20, 40). EO MAY INHIBIT Na+/K+-ATPASE IN THE CHOROID PLEXUS (23) AND, THEREBY, ATTENUATE Na+ ENTRY INTO THE CSF (17). ALL 3 α-ISOMERS OF Na+/K+-ATPASE ARE ALSO EXPRESSED IN HYPOTHALAMIC NUCLEI, SUCH AS THE PARAVENTRICULAR (PVN) AND SUPRAOPTIC (SON) NUCLEI (26). MICROINJECTION OF OUAHIN INTO THE PVN CAUSES DOSE-RELATED INCREASES IN MEAN ARTERIAL PRESSURE (MAP) AND HEART RATE (HR), WHICH CAN BE PREVENTED BY AN AT1 RECEPTOR BLOCKER (7). Thus, EO may inhibit Na+/K+-ATPase in the membrane of neurons, increasing intracellular Ca2+ (30), and thereby enhance activity of angiotensinergic sympathoexcitatory pathways leading to hypertension (7).

Salt-inducible kinase 1 (SIK1) has been shown to regulate aldosterone synthesis, as well as intracellular [Na+] (38). SIK1 represses gene expression of CYP11A, CYP11B1, and CYP11B2, and attenuates ACTH-induced steroidogenesis in the adrenal cortex (36, 38). SIK1 also is part of a cell sodium-sensing network that regulates active sodium transport by Na+/K+-ATPase in a calcium-dependent process in kidney cells (32). The SIK1 gene is located within a MAP quantitative trait locus (QTL) on chromosome 20p12, which may contribute to salt-induced hypertension in Milan hypertensive rats, Na+/K+-ATPase activity in proximal tubules is increased via activation of the SIK1-Na+/K+-ATPase network (28, 33). Basal SIK1 phosphorylation and sodium transport were increased in a cell line of proximal tubule origin by a hypertension-linked mutation of human α-adducin (33).

SIK1 has also been identified in rat brain, and SIK1 mRNA increases up to eight-fold in the hippocampus and cortex following depolarization (6). We hypothesized that the SIK1-Na+/K+-ATPase network in the choroid plexus or neuronal membrane contributes to regulation of CSF [Na+] and hypothalamic aldosterone and neuronal excitability, and dysregulation of this SIK1- Na+/K+-ATPase network in the brain contributes to salt-induced hypertension.

In the present study, we examined whether SIK1 in the brain contributes to 1) increases in Na+ entry into CSF and in hypothalamic aldosterone in Dahl S rats on a high-salt diet; and 2) excitatory MAP, HR, and sympathoexcitatory responses to CSF [Na+]. To assess the role of SIK1, we employed central administration of staurosporine. Staurosporine inhibits SIK1 activity at 5–10 nM (2.4–4.7 ng/ml, with mol wt = 467) in vitro (22, 32), and at higher concentrations, it inhibits other kinases, e.g., PKC with IC50 of ~50 nM (2). We assessed 1) SIK protein and activity in hypothalamic tissue of Dahl S and salt-resistant consomic SS.BN13 rats, and the concentrations of staurosporine needed to inhibit SIK1 activity; 2) effects of intracerebroventricular staurosporine on excitatory MAP, HR, and renal sympathetic nerve activity (RSNA) responses to intracerebroventricular infusion of Na+-rich aCSF in Dahl S, SS.BN13, and Wistar rats; 3) effects of chronic intracerebroventricular infusion of staurosporine on CSF [Na+], resting MAP, and HR, and hypothalamic aldosterone and corticosterone content in Dahl S and SS.BN13 rats on a high-salt diet for 2 wk; and 4) as “control” studies, effects of intracerebroven-
tricular staurosporine on responses to intracerebroventricular ANG II and carbachol, and of the specific PKC inhibitor GF109203X on pressor responses to intracerebroventricular Na+/rich aCSF and intracerebroventricular ANG II.

**METHODS**

Five- to six-week-old male Dahl S rats (SS/Mcw) and salt-resistant consomic (SS.BN13) rats, and Wistar rats were purchased from Charles River Laboratories (Montreal, Canada). SS.BN13 rats show functional and histological responses to a high-salt diet, which are similar to normotensive strains (4) and are used as salt-resistant control for Dahl S rats (4, 11). The animals were housed on a 12:12-h light-dark cycle and fed a standard commercial rat chow with normal salt intake (101 μmol Na+/g; Harlan Laboratories, Madison, WI; cat. no. TD.2019) and tap water ad libitum. All experiments were approved by the University of Ottawa Animal Care Committee and conform with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996).

**Effects on responses of MAP, HR, and RSNA.** Dahl S and SS.BN13 and Wistar rats (two groups/strain, n = 5–8/group) remained on normal salt intake. Under isoflurane anesthesia, a 23-gauge stainless-steel cannula was placed just above the right lateral cerebroventricle as a guide cannula and fixed on the skull with dental cement (19). At least 5 days following the head surgery, the rats were anesthetized with isoflurane, and the right femoral artery and vein were cannulated with PE-10/PE-50 tubings, which were tunneled to the back of the neck. Through a flank incision, a pair of silver electrodes was then placed around and fixed to the left renal nerve with silicone rubber (Wacker Sil-Gel, 604 A B, Wacker, Munich, Germany) and exteriorized, as described previously (19).

At least 4 h after recovery from the anesthesia in the original cage, the intra-arterial catheter was connected to a transducer to record MAP and HR with a Grass polygraph (19). The electrodes were connected to a high-impedance probe, and the electrical signals were amplified 20,000–50,000 times by a band-pass amplifier (Grass P511, Grass Technologies/Astro-Med, West Warwick, RI) with a high- and low-frequency cutoff of 1,000 and 30 Hz, respectively. The filtered signals were channeled to a rectifying voltage integrator (Grass 7P10) and recorded through the Grass polygraph. The RSNA signal (in millivolts), together with MAP and HR, were also fed into an on-line computer with a Grass data acquisition and analysis program (Polyview 2.0). The signal noise for RSNA was determined after the rat had been killed at the end of the study and subtracted from the total activity (19).

After a 30-min rest, animals underwent baseline MAP, HR, and RSNA recordings in unrestrained animals for 10 min. A 26-gauge stainless-steel needle was then inserted into the guide cannula, so that its tip protruded into the lateral cerebroventricle. Via a PE tubing, the other end of the needle was connected to a 500-μl Hamilton microsyringe, which was mounted on a Sage 355 infusion pump for intracerebroventricular infusion. Following a 20-min rest, animals were infused with Na+/rich aCSF (300 mmol/l Na+) at 3.8 μl/min for 8 min. About 20 min after responses had subsided, staurosporine (5 ng/2 μl) or vehicle (2 μl aCSF with 0.5% ethanol) was injected intracerebroventricularly in two groups of rats for each strain. After 3 min, intracerebroventricular infusion of Na+/rich aCSF was repeated at the same rate for 8 min. Five minutes before each intracerebroventricular infusion, the vasopressin V1 receptor antagonist d(CH2)5-Tyr(Me)AVP (30 μg/kg in 0.1–0.2 ml saline; Sigma Chemical, St. Louis, MO) was injected intravenously, to exclude the effects of vasopressin release by the Na+/rich aCSF. Intravenous injection of the vasopres- sin antagonist did not affect resting MAP, RSNA, and HR.

**Effects on CSF [Na+].** In a second set of 12 Wistar rats, an intracerebroventricular cannula was inserted as described above. In addition, a hole was drilled in the skull on the sagittal midline immediately rostral to the interparietal-occipital bone suture for CSF withdrawal from the cisterna magna. The rats were divided into three groups: 1) intracerebroventricular injection of vehicle then intracerebroventricular infusion of aCSF (n = 4); 2) intracerebroventricular injection of vehicle then intracerebroventricular infusion of Na+/rich aCSF (n = 4); 3) intracerebroventricular injection of staurosporine then intracerebroventricular infusion of Na+/rich aCSF (n = 4). Via a microsyringe, staurosporine (5 ng/2 μl) or vehicle (2 μl aCSF with 0.5% ethanol) was injected intracerebroventricularly under anesthesia. After 0.5 min, aCSF or Na+/rich aCSF (300 mmol/l Na+) was infused intracerebroventricularly at 3.8 μl/min for 20 min. About 30 s before the end of the intracerebroventricular infusion, a 25-gauge, Pencan pencil point spinal needle (B. Braun Medical) was inserted into the hole above the cisterna magna at a 70–75° angle to the skull surface and was advanced about 7.5 mm. With an 1-ml syringe, 150–200 μl of CSF was withdrawn at <10 μl/s, for measurement of [Na+] with a Na+-sensing electrode (model MI-425; Microelectrodes, Bedford, NH).

**Effects of intracerebroventricular injection of staurosporine on responses to intracerebroventricular injection of ANG II and carbachol.** In 12 Wistar rats, effects of intracerebroventricular injection of staurosporine on responses to intracerebroventricular injection of ANG II or carbachol were tested. Rats were instrumented with an intracerebroventricular guide cannula and an intra-arterial catheter in the femoral artery. Four hours after recovery from the anesthesia for arterial cannulation, MAP and HR responses to intracerebroventricular injection of ANG II (n = 6) or cholinergic mimetic carbachol (n = 6), each at two doses (15), were examined. Following a 20-min rest, 30 ng/2 μl ANG II or 25 ng/2 μl carbachol was injected intracerebroventricularly. After the MAP and HR responses had reached a plateau, 90 ng/2 μl ANG II or 75 ng/2 μl carbachol was injected. About 20 min after responses had subsided, staurosporine (5 ng/2 μl) was injected intracerebroventricularly. Three minutes later, the two doses of ANG II or carbachol were repeated. ANG II and carbachol were purchased from Sigma and dissolved in aCSF.

**Effects of intracerebroventricular injection of PKC inhibitor GF109203X on responses to intracerebroventricular Na+/rich aCSF and ANG II.** GF109203X is a highly selective, cell-permeable PKC inhibitor with a similar structure to staurosporine. In vitro, the effective concentration of GF109203X is 1 μM (34) or 0.4 μg/ml. In rats, microinjection of GF109203X into the NTS at 0.08 μg/ml inhibits ANG II-induced inhibition of baroreflex gain (34). Considering the production rate and total volume of CSF in rats, we estimated that the dose for acute intracerebroventricular administration needs to be at least 0.2 μg to inhibit PKC in the brain. Two groups (n = 8/group) of Wistar rats on a normal-salt diet were instrumented with an intracerebroventricular guide cannula, as well as an intra-arterial catheter in the femoral artery 5 days later. About 18 h after arterial cannulation, MAP and HR were recorded for 10 min in resting rats, and via a stainless-steel needle Na+/rich aCSF was infused intracerebroventricular at 3.8 μl/min for 8 min. About 20 min after responses had subsided, ANG II (25 and 75 ng/2 μl) was injected intracerebroventricularly. Twenty minutes after the responses had subsided, the selective PKC inhibitor GF109203X (0.5 μg/μl; Cayman Chemical) or vehicle (4 μl aCSF with 2% DMSO) was injected. After 3 min, intracerebroventricular infusion of Na+/rich aCSF and injection of ANG II were repeated.

**Effects of chronic intracerebroventricular infusion of staurosporine in Dahl rats.** In two groups of Dahl S rats and two groups of SS.BN13 rats (n = 7 or 8/group) under isoflurane anesthesia, an L-shaped, 23-gauge stainless-steel cannula was placed into the right lateral cerebroventricle (14). Via polyethylene tubing, the other end of the cannula was connected to an osmotic minipump (Alzet, Dyrect, Cupertino, CA; model 2002; rate: 0.5 μl/h) placed subcutaneously for
intracerebroventricular infusion of either staurosporine (Sigma Chemical) at 25 ng/day or vehicle (aCSF with 0.5% ethanol). In our previous studies, intracerebroventricular infusion of aCSF with 1–2% ethanol at the same rate had no effects on resting hemodynamics or behavior (37). The rate for intracerebroventricular infusion of staurosporine was estimated from in vitro studies, and the total volume and production rate of CSF. Considering that the CSF production rate is about ~6 ml/day and total CSF volume is ~0.5 ml in rats of ~300 g body wt (5, 10), intracerebroventricular infusion of staurosporine at 25 ng/day may result in a CSF concentration of ~4 ng/ml (25 ng/6 ml) or 8.5 nM. Intravenous or subcutaneous rates are approximately 30 mg/kg·day−1 (41), and it is, therefore, unlikely that the intracerebroventricular infusion at 25 ng/day would cause relevant peripheral effects. A high-salt diet (1,370 μmol Na+; Harlan Laboratories; cat. no. TD.79119) was started in all four groups following the surgery and lasted for 2 wk.

At the end of the 2-wk intracerebroventricular infusion and high-salt intake, under isoflurane anesthesia, a PE catheter (PE-10 fused to PE-50) was placed into the right femoral artery. The catheter was filled with heparinized saline and exteriorized on the back of each rat. The next morning, about 18 h after the arterial cannulation, the arterial line was connected to a pressure transducer. Grass polygraph, and tachograph. After a rest of about 30 min, MAP and HR were recorded for 5 min. The average MAP and HR recorded over these 5 min were used as the resting MAP and HR. The rat was then reanesthetized with isoflurane, and 100–200 μl CSF was collected from the cisterna magna at ≤10 μl/s (14). After decapitation, the whole brain was removed, frozen quickly in dry ice, and stored at −80°C. The whole hypothalamus and hippocampus were dissected according to Glowinski and Iversen (8).

The accuracy of the placement of the intracerebroventricular cannulas was verified by either injection of blue dye after the short-term study or by visual examination during tissue collection in the chronic study. A few rats (3 out of 61) with unsuccessful intracerebroventricular cannula placement in the acute experiments were excluded.

SIK1 protein and activity in the hypothalamus. SIK1 activity was measured in immunopurified hypothalamic tissue, using [γ-32P]-ATP (Perkin Elmer, Wellesley, MA; 10 Ci/mmol 2 mM/Ci/ml), the synthetic peptide substrate syntide-2 (6), (product no. sc-201151; Santa Cruz Biotecnology, Santa Cruz, CA), and the phosphocellulose binding technique (3). Briefly, the hypothalami from four Dahl SS.BN13 and 4 Dahl S rats were first homogenized in lysis buffer, centrifuged at 12,000 g for 20 min at 4°C; then replicate aliquots of the supernatants (containing 250 μg total protein) were prepared. After immunoprecipitation with anti-SIK1 and protein A/G + agarose (product nos. sc-83754 and sc-2003, respectively; Santa Cruz Biotechnology), the purified SIK1 was resuspended in 55 μM Tris·HCl pH 7.2, 50 mM MgCl2, 4 mM EDTA, 10 mM EGTA, 25 mM β-glycercophosphate, with 0.5 mM DTT added freshly, and 17.5 μl was added to an equal volume of reagent mixture containing 1.2 μCi [γ-32P]-ATP, 200 μg BSA, and 10 μl substrate (1 mg/ml) in ddH2O on ice. After 20 min of incubation, at 30°C, an aliquot of each reaction mixture was spotted onto a labeled P81 cellulose phosphate paper disc, which was immediately washed 5 × 5 min in 75 mM H3PO4, then counted (3 min) in CytoScint-ES cocktail (no. 882453; MP Biomedicals, Santa Ana, CA) using a Packard Tri-Carb 2100TR LS Analyzer. SIK1 activity was directly proportional to the cpm, a measure of the [γ-32P] incorporated into the synthetic substrate. A known amount of ATP (25 ng) was added to one of the replicates for each sample to calculate the actual SIK1 activity in that sample.

To assess the in vitro inhibition of SIK1 by staurosporine, SIK1 activity was compared in replicate aliquots of the immunopurified tissue preparations to which staurosporine was added in final concentrations of 0, 5, 25, or 50 nM (determined from initial experiments with active SIK1; Signal Chem no. S14–11H-05). The activity in the samples with added staurosporine was expressed as a percentage of that in the same sample without the inhibitor.

In a separate experiment, the identity of the proteins in the immunoprecipitates was confirmed by SDS-PAGE using a 10% bis-acrylamide gel. Proteins were transblotted onto a PVDF membrane, which was subsequently blocked with 5% milk, incubated at 4°C overnight in rabbit anti-SIK1 antibody (product no. sc-83754, 1:500 dilution; Santa Cruz Biotechnology), washed, and then incubated at room temperature for 1 h in goat anti-rabbit HRP-conjugated secondary antibody (no. sc-2004, 1:5000; Santa Cruz Biotechnology). Proteins were visualized with ECL Western Lightning Plus reagents (no. NEL104001; Perkin Elmer) and an Alpha-Ease imaging system. For immunoblotting of the total lysates from the same samples, the membrane was reprobed with β-actin antibody (no. A2228, 1:10,000; Sigma), for normalization of the SIK1 bands. Specific bands, which were completely eliminated by preincubation of the primary antibody with an excess of the immunizing peptide, were observed at ~85 and 35 kDa (6, 22, 32).

Aldosterone and corticosterone in hypothalamus and hippocampus. Aldosterone was measured by radioimmunoassay (RIA) after tissue preparation and Sep-Pak extraction as described previously (25) using a rabbit antiserum and [3H]-labeled aldosterone (nos. 07–108216 and 07–108226; MP Biomedicals). The sensitivity for aldosterone was 35 pg/g in hypothalamic tissue (12), and 17 pg/g in the hippocampus. All samples from the experiment were done in one assay, with an intra-assay variation of 7%. Cross reactivities were 0.03 and 0.14% for corticosterone and DOC, respectively.

For measurement of corticosterone, the brain tissues were prepared as for aldosterone, then the Sep-Pak eluates were further diluted with steroid diluent (no. 07–166197; MP Biomedicals) before assay using a corticosterone 121I RIA kit (no. 07–120103; MP Biomedicals), according to the manufacturer’s instructions. The sensitivity for hypothalamic corticosterone was 0.71 ng/g, and for the hippocampus, it was 0.33 ng/g. The corticosterone antibody had 0.34% cross reactivity with desoxycorticosterone, and 0.1% with testosterone.

Recovery for the Sep-Pak extraction, measured by spiking with known concentrations, was similar for both aldosterone and corticosterone at ≥88%. Reagent blanks for the Sep-Pak extraction and subsequent manipulations showed not significant readings.

Data analysis. Values are expressed as means ± SE. Responses of RSNA were expressed as percentages of resting values. For the chronic study and maximal responses in acute studies, two-way ANOVA was performed. For RSNA, MAP and HR responses to acute intracerebroventricular infusion of Na+-rich aCSF, one-way or two-way repeated-measures ANOVA was performed. For CSF [Na+] after acute infusion of Na+-rich aCSF, one-way ANOVA was performed. In vitro percent inhibition of SIK1 activity was analyzed by two-way ANOVA. For all of these tests, when the F values were significant for main effect, Bonferroni t-test was used for multiple comparisons. Comparisons for acute responses before vs. after staurosorpin (both absolute and percentage changes) were analyzed with paired t-test. Statistical significance was defined as P < 0.05.

RESULTS

Hypothalamic SIK1 protein and activity, and inhibition by staurosporine. For SIK1 protein, both bands were significantly lower by 20–30% in the hypothalamus of the Dahl S rats compared with the SS.BN13 controls (Fig. 1). SIK1 activity in the hypothalamus followed the same pattern (Fig. 1). Staurosporine at 5, 25, and 50 nM inhibited hypothalamic SIK1 activity by 50–80% in a dose-related manner in both SS.BN13 and S rats (Fig. 2). The in vitro percent inhibition by staurosporine at corresponding concentrations was somewhat more pronounced in the S rats (P = 0.04).

Intracerebroventricular staurosporine and responses to acute increases in CSF [Na+]. Among Wistar, SS.BN13, and Dahl S rats on a normal-salt diet, there were no significant differences in resting MAP (109 ± 4, 107 ± 3, and 114 ± 4
mmHg), HR (398 ± 14, 387 ± 12, and 409 ± 10 bpm), and RSNA (6.7 ± 2.2, 5.6 ± 2.0 and 7.7 ± 2.1 mV). MAP, HR, and RSNA started to increase within 1 or 2 min after the start of intracerebroventricular infusion of Na⁺-rich aCSF, reached a plateau within 3 or 4 min, and returned to basal levels within 2 or 3 min after the termination of intracerebroventricular infusion (Figs. 3 and 4). This pattern was similar in Wistar and SS.BN13 and S rats, except that the maximal responses were significantly larger in Dahl S rats (Table 1, Fig. 4).

No changes in resting MAP, HR, and RSNA were observed during the 3 min after intracerebroventricular injection of vehicle or staurosporine (data not shown). Na⁺-rich aCSF-induced increases in MAP, HR, and RSNA were not affected by intracerebroventricular vehicle in all three strains (Figs. 3 and 4). In contrast, after intracerebroventricular staurosporine, the maximal increases in MAP, HR, and RSNA induced by intracerebroventricular infusion of Na⁺-rich aCSF were significantly larger compared with those before staurosporine (Table 1, Figs. 3 and 4). Whereas the absolute differences between maximal MAP responses after vs. before staurosporine were similar in the three strains, the differences of RSNA and HR responses were significantly smaller in Dahl S vs. SS.BN13 or Wistar rats (Table 1). However, the percent increases in the maximal responses to intracerebroventricular infusion of Na⁺-rich aCSF remaining significantly larger after staurosporine in Dahl S rats vs. SS.BN13 or Wistar rats (Table 1).

Compared to intracerebroventricular infusion of aCSF, intracerebroventricular infusion of Na⁺-rich aCSF for 20 min significantly increased CSF [Na⁺] (173 ± 5 vs. 154 ± 1 mmol/l, P < 0.05). Intracerebroventricular injection of staurosporine did not affect this increase in CSF [Na⁺] induced by intracerebroventricular infusion of Na⁺-rich aCSF (170 ± 6 vs. 173 ± 5 mmol/l in rats with intracerebroventricular injection of vehicle).

Intracerebroventricular staurosporine and responses to intracerebroventricular ANG II and carbachol. As shown in Table 2, intracerebroventricular injection of ANG II increased MAP and HR in a dose-related manner, and intracerebroventricular

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**Fig. 1.** SIK1 protein (left) and activity (right) in the hypothalamus of Dahl SS.BN13 and S rats after 2 wk on a high-salt diet. Bottom: specific bands, eliminated by preincubation of the antibody with immunizing peptide, were detected at ~85 and 36 kDa. Band densities, in arbitrary units, were normalized to β-actin as a loading control. For the activity assay, activity was measured in duplicate aliquots of each purified sample, after the addition of 25 ng SIK1 to one aliquot and is expressed as nanogram equivalents. Data are expressed as means ± SE, as analyzed with Student’s t-test. (n = 4/group for immunoblotting; n = 7 for SS.BN13 and n = 5 for Dahl S for the activity assay). *P < 0.05 between strains.

**Fig. 2.** In vitro percent inhibition of SIK1 activity by staurosporine at 5–50 nM in hypothalamic tissue of Dahl S and SS.BN13 rats. Data are expressed as means ± SE (n = 4/ per concentration), expressed as percent activity of corresponding control tissue and analyzed with two-way ANOVA. Between strains: F = 4.57, P = 0.04; between treatments: F = 109.50, P < 0.0001; between strains × treatments: F = 0.555, P = 0.64. *P < 0.05, vs. control; *P < 0.05, vs. 5 or 25 nM.
tricular injection of vehicle or GF109203X. Na\(^{+}\) increased MAP and decreased HR in a dose-related fashion, and these responses were also not affected by staurosporine. Intracerebroventricular injection of carbachol increased MAP and decreased HR in a dose-related fashion, and these responses were also not affected by staurosporine.

**Effects of GF109203X on responses to intracerebroventricular Na\(^{+}\)-rich aCSF and ANG II.** No changes in resting MAP and HR were observed during the 3 min after intracerebroventricular injection of vehicle or GF109203X. Na\(^{+}\)-rich aCSF or ANG II-induced increases in MAP were similar before and after intracerebroventricular injection (Fig. 5). In contrast, after intracerebroventricular infusion of GF109203X, the increases in MAP by Na\(^{+}\)-rich aCSF or ANG II were significantly attenuated by 50–70\% compared with those after injection of vehicle or before intracerebroventricular GF109203X.

**Chronic intracerebroventricular infusion of staurosporine in Dahl rats on a high-salt diet.** There were no significant differences in initial body weight or weight gain among the four groups of rats on a high-salt diet, treated with either intracerebroventricular vehicle or staurosporine for 2 wk (228 ± 12 and 210 ± 9 g or +58 ± 3 and +61 ± 4 g for Dahl S and SS.BN13 rats with vehicle, and 225 ± 13 and 208 ± 13 or +59 ± 4 and +63 ± 3 g for Dahl S and SS.BN13 rats with staurosporine).

After 2 wk of a high-salt diet, Dahl S rats treated with vehicle had a significantly higher resting MAP than SS.BN13 rats treated with vehicle (Fig. 6). In SS.BN13 rats, MAP was not affected by intracerebroventricular infusion of staurosporine. In contrast, in Dahl S rats intracerebroventricular infusion of staurosporine further increased MAP significantly by ~15 mmHg. Resting HR tended (\(P = 0.07\)) to be higher in Dahl S vs. SS.BN13 rats treated with intracerebroventricular infusion of vehicle and was significantly higher in Dahl S rats with intracerebroventricular infusion of staurosporine.

After 2 wk of a high-salt diet, CSF [Na\(^{+}\)] was significantly higher by 5 mmol/l in Dahl S vs. SS.BN13 rats treated with vehicle (Fig. 6). Staurosporine did not affect the high-salt diet-induced increase in CSF [Na\(^{+}\)] observed in Dahl S rats.

Compared with SS.BN13 rats, Dahl S rats on a high-salt diet showed significantly higher aldosterone levels in both the hypothalamus and hippocampus. Corticosterone levels were only increased in the hypothalamus (Fig. 7). These increases persisted in Dahl S rats treated with intracerebroventricular infusion of staurosporine (Fig. 7).

**DISCUSSION**

The present study shows that 1) SIK1 protein and activity are significantly lower in the hypothalamus of Dahl S vs. SS.BN13 rats; 2) intracerebroventricular injection of staurosporine significantly enhances excitatory RSNA, MAP, and HR responses to intracerebroventricular infusion of Na\(^{+}\)-rich aCSF; the extent of this enhancement is significantly smaller for RSNA and HR in Dahl S rats compared with SS.BN13 and Wistar rats; 3) intracerebroventricular infusion of staurosporine at a rate of 25 ng/day significantly enhances salt-induced increases in resting MAP and HR without affecting the increases in CSF [Na\(^{+}\)] and hypothalamic aldosterone in Dahl S rats on a high-salt diet; and 4) intracerebroventricular injection of staurosporine does not affect MAP and HR responses induced by intracerebroventricular ANG II or carbachol, whereas intracerebroventricular injection of a specific PKC inhibitor markedly inhibits pressor responses to intracerebroventricular Na\(^{+}\)-rich aCSF or ANG II.

An increase in intracellular [Na\(^{+}\)] leads to activation of Na\(^{+}\)/K\(^{+}\)-ATPase to increase outward Na\(^{+}\) transport. SIK1 may serve as part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process (32). An increase in intracellular [Na\(^{+}\)] may increase intracellular [Ca\(^{2+}\)] through the Na\(^{+}\)/Ca\(^{2+}\) exchanger, leading to SIK1 activation. The latter results in dephosphorylation of the Na\(^{+}\)/K\(^{+}\)-ATPase \(\alpha\)-subunit and an increase in its activity (32). Considering its peripheral actions, in the CNS, SIK1 may potentially affect several components of the pathways leading from increased CSF [Na\(^{+}\)] to sympathoexcitation and hypertension. To assess the role of SIK1 in the CNS, we used the kinase inhibitor staurosporine. First, intracerebroventricular staurosporine did not affect the increase in CSF [Na\(^{+}\)] in Dahl S rats on a high-salt diet. The choroid plexus is the major site for production of CSF (29), and Na\(^{+}\)/K\(^{+}\)-ATPase plays a major role in transporting sodium into the CSF (1, 17). The absence of changes in CSF [Na\(^{+}\)] with chronic intracerebroventricular infusion of staurosporine in Dahl S and SS.BN13 rats on a high-salt diet suggests that in contrast to the renal tubules (33), SIK1 per se may play, at most, a minor functional
role in Na\(^+\) transport across the choroid plexus. In addition, this finding suggests that the increase in CSF [Na\(^+\)] in Dahl S rats on a high-salt diet is not due to increased SIK1 activity in the choroid plexus.

Secondly, chronic intracerebroventricular infusion of staurosporine did not affect the increase in hypothalamic aldosterone and corticosterone in Dahl S rats on a high-salt diet. Since central infusion of an aldosterone synthase inhibitor prevents the increase in aldosterone (20), it appears that an increase in CSF [Na\(^+\)] plays a functional role in regulation of hypothalamic aldosterone and corticosterone synthesis in response to an increase in CSF [Na\(^+\)]. However, possible effects of staurosporine on aldosterone and corticosterone levels in specific hypothalamic nuclei cannot be excluded.

Table 1. Maximal increases in MAP, RSNA, and HR by intracerebroventricular infusion of Na\(^+\)-rich aCSF before and after intracerebroventricular staurosporine in Wistar, Dahl S, and SS.BN13 rats

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>RSNA, %</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Increase</td>
</tr>
<tr>
<td>Wistar</td>
<td>11 ± 1</td>
<td>19 ± 2</td>
<td>+8 ± 1 (+70 ± 2%)</td>
</tr>
<tr>
<td>SS.BN13</td>
<td>14 ± 2</td>
<td>23 ± 2</td>
<td>+9 ± 1 (+69 ± 3%)</td>
</tr>
<tr>
<td>Dahl S</td>
<td>29 ± 1*</td>
<td>36 ± 3*</td>
<td>+7 ± 2 (+20 ± 2%)*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE (for n, see Figs. 3 and 4) and analyzed with two-way ANOVA. Percent changes in brackets represent percent increases in responses to Na\(^+\)-rich artificial cerebrospinal fluid (aCSF) after intracerebroventricular staurosporine (5 ng) within strains. For comparisons of maximal responses between strains either before or after staurosporine: for mean arterial pressure (MAP), F = 68.1 (P = 0.0001); for renal sympathetic nerve activity (RSNA), F = 25.5 (P = 0.0002); and for heart rate (HR) F = 30.1 (P = 0.0002). For comparisons of maximal responses before and after staurosporine in all strains: for MAP, F = 40.1 (P = 0.0001); for RSNA, F = 49.9 (P = 0.0001); and for HR, F = 31.9 (P = 0.0001). For comparisons of absolute increases in responses: for MAP, F = 0.58 (P = 0.56); for RSNA, F = 16.0 (P = 0.0001); and for HR, F = 22.5 (P = 0.0001). For comparisons of % increases in responses: for MAP, F = 51.7 (P = 0.001); for RSNA, F = 43.3 (P = 0.001); and for HR, F = 33.6 (P = 0.001). Maximal responses after staurosporine are all significantly (P < 0.05) increased compared with those before staurosporine. *P < 0.05, vs. SS.BN13 and Wistar rats either before or after staurosporine. **P < 0.05, vs. SS.BN13 and Wistar rats.
In contrast to the above “negative” results, intracerebroventricular staurosporine markedly enhanced sympathoexcitatory and pressor responses to an increase in CSF [Na\(^+\)]. Intracerebroventricular infusion of Na\(^+\)-rich aCSF caused parallel increases in RSNA, MAP, and HR, and these responses were significantly enhanced by 75\% with intracerebroventricular injection of staurosporine in Wistar or SS.BN13 rats. One may speculate that a sodium-induced activation of neuronal SIK1 and, thereby, Na\(^+\)/K\(^+\)-ATPase attenuates sodium-induced sympathoexcitatory and pressor responses. Removal of this compensatory mechanism by inhibiting SIK1 with staurosporine unmasks the “full” sympathoexcitatory and pressor responses to CSF Na\(^+\). Intracerebroventricular staurosporine did not affect the increase in CSF [Na\(^+\)] caused by the intracerebroventricular infusion of Na\(^+\)-rich aCSF, making it unlikely that the enhanced responses are due to larger increases in CSF [Na\(^+\)]. Alternatively staurosporine affects several kinases in a concentration-dependent manner (2, 22) and may cause a generalized enhancement of sympathoexcitatory responses. This is unlikely. First, estimated CSF concentrations for staurosporine in the present study are \(\sim 4\) ng/ml, or 8.5 nM. As
shown in Fig. 2, at this concentration, staurosporine inhibits SIK1 activity by 50–60%. This IC$_{50}$ is consistent with previous studies (22, 32). Higher concentrations are needed to inhibit other kinases, with e.g., an IC$_{50}$ of $\sim$50 nM for PKC (2). Secondly, inhibition of these kinases would be expected to attenuate responses to intracerebroventricular Na$^+$ rather than enhance responses. Sympathoexcitatory and pressor responses to intracerebroventricular Na$^+$-rich aCSF or a high-salt diet in Dahl S rats are mediated by angiotensinergic sympathoexcitatory pathways and can be blocked by intracerebroventricular infusion of an AT$_1$-receptor blocker (16, 18, 35). Activation of several kinases, such as PKC, is involved in ANG II signaling in the central nervous system (9, 39). Staurosporine did not affect the pressor responses induced by central AT$_1$ receptor stimulation with intracerebroventricular ANG II. Responses to central muscarinic receptor stimulation with carbachol, which also involve activation of PKC, were also not affected by staurosporine. In contrast, the selective PKC inhibitor GF109203X inhibited 60–70% of the pressor responses to both intracerebroventricular Na$^+$-rich aCSF and intracerebroventricular ANG II. Similarly, intracerebroventricular infusion of a selective inhibitor of p44/42 mitogen-activated protein kinase also markedly attenuates increases in MAP, RSNA, and neuronal activity in the PVN induced by intracerebroventricular injection of ANG II (39).

Excitatory RSNA, HR, and MAP responses to intracerebroventricular Na$^+$-rich aCSF are significantly larger in Dahl S vs. SS.BN13 or Wistar rats. After staurosporine, all these responses were increased in the three strains, but both absolute and percent increases in RSNA and HR and percent increase in MAP responses were significantly less in Dahl S vs. SS.BN13 and Wistar rats. The absolute increases in MAP responses by staurosporine were similar in the Dahl S rats compared with the two controls, possibly reflecting enhanced arterial responses in Dahl S rats. These findings suggest that the neuronal SIK1 network is less effective in Dahl S rats, and as a result, its inhibition by staurosporine leads to smaller increases in the RSNA, MAP, and HR responses to CSF Na$^+$. These functional studies do not clarify which components of the SIK1-Na$^+$/K$^+$-ATPase network are different or differently regulated in Dahl S vs. SS.BN13 and Wistar rats. In the whole hypothalamus, SIK1 protein and enzymatic activity were significantly lower in Dahl S vs. SS.BN13 rats. On the other hand, inhibition of enzymatic activity by staurosporine in vitro was somewhat larger in Dahl S rats. These findings may indicate that expression of SIK1 per se is decreased in Dahl S rats and that in vivo regulators of SIK1 protein expression may be less active in Dahl S rats compared with the control strains.

Chronic intracerebroventricular infusion of staurosporine significantly increased MAP and HR only in Dahl S and not SS.BN13 rats on a high-salt diet. Since the increase in CSF [Na$^+$] in Dahl S rats on a high-salt diet was not affected by intracerebroventricular infusion of staurosporine, it appears that the further increase in neuronal responsiveness to CSF Na$^+$ by staurosporine contributes to this larger increase in MAP and HR in Dahl S rats on a high-salt diet. This finding also suggests that the neuronal SIK1 network, although less effective, still plays a buffering role in Dahl S rats in attenuating the hypertension. Chronic intracerebroventricular infusion of staurosporine had no effect on the MAP of SS.BN13 rats on a high-salt diet. CSF [Na$^+$] does not increase in Dahl salt-resistant (R) rats with high salt intake (17), and an increase in CSF/interstitial [Na$^+$] appears to be needed to activate the neuronal SIK1-Na$^+$/K$^+$-ATPase network and for inhibition by staurosporine to have an effect. Supporting this conclusion, acute injection of staurosporine also did not affect resting RSNA, MAP, and HR in all three strains.

After intracerebroventricular staurosporine, the absolute MAP, RSNA, and HR responses to intracerebroventricular Na$^+$-rich aCSF were still larger in Dahl S rats vs. the other two
strains, suggesting that other mechanisms also contribute to increased neuronal responsiveness to CSF Na\(^+\) in Dahl S rats. We previously demonstrated (13) that in Dahl S rats, variants within C10 QTL2 on chromosome 10 contribute to gain-of-function of central mechanisms determining neuronal responses to CSF [Na\(^+\)]\(^+\). Whether these mechanisms interact with the SIK1-Na\(^+\)/K\(^+\)-ATPase network will require further study.

Limitations. There are some limitations to consider. Firstly, intracerebroventricular infusion and whole hypothalamus assays do not provide insights into the central pathways/nuclei where SIK1 is operative. Local injections of staurosporine and assays in punches of nuclei may be useful in this regard. Secondly, food (and salt) intake were not measured, and an increase in MAP could result from increased salt and water consumption by staurosporine. However, the gain of body weight and visible food intake on a high-salt diet and CSF [Na\(^+\)]\(^+\) were similar in Dahl S rats on a high-salt diet with and without intracerebroventricular infusion of staurosporine, and no behavioral changes were observed in staurosporine-treated rats. It appears unlikely that staurosporine itself further increased salt intake and thereby increased MAP. Thirdly, MAP and HR were measured through an arterial catheter 18 h after cannulation and a 30-min rest. These are not as ideal as data obtained by telemetry, and likely overestimate resting values somewhat.

Perspectives and Significances

The present study provides new insights into intraneuronal mechanisms regulating neuronal responses to CSF Na\(^+\) and the resulting sympathoexcitation and salt-sensitive hypertension. Neuronal SIK1 -Na\(^+\)/K\(^+\)-ATPase network appears to play an important functional role in the responses to increases in brain [Na\(^+\)]\(^+\) so as to attenuate CSF Na\(^+\)-induced sympathoexcitatory and pressor responses. The lower hypothalamic SIK1 protein activity and lower effectiveness of this buffering mechanism in Dahl S rats suggest that variations in the neuronal SIK1-Na\(^+\)/K\(^+\)-ATPase network, possibly variants in the SIK1 gene (22, 31), may contribute to salt sensitivity in Dahl S rats.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: B.S.H. and F.H.L. conception and design of research; B.S.H. and R.A.W. performed experiments; B.S.H. and R.A.W. analyzed data; B.S.H. and F.H.L. interpreted results of experiments; B.S.H. prepared figures; B.S.H. drafted manuscript; B.S.H., R.A.W., and F.H.L. edited and revised manuscript; B.S.H. and F.H.L. approved final version of manuscript.

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