Cardiac sympathetic afferent stimulation induces salt-sensitive sympathoexcitation through hypothalamic epithelial Na$^+$ channel activation

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Ito K, Hirooka Y, Sunagawa K. Cardiac sympathetic afferent stimulation induces salt-sensitive sympathoexcitation through hypothalamic epithelial Na$^+$ channel activation. Am J Physiol Heart Circ Physiol 308: H530–H539, 2015. First published December 19, 2014; doi:10.1152/ajpheart.00586.2014.—The cardiac sympathetic afferent (CSA), which plays an important role in heart-brain communication for sympathoexcitation, is stimulated in heart failure. Additionally, high salt intake leads to further sympathoexcitation due to activation of hypothalamic epithelial Na$^+$ channels (ENaCs) in heart failure. In the present study, we stimulated the CSA in adult male mice by epicardial application of capsaicin and using ethanol as a control to determine whether CSA stimulation led to activation of hypothalamic ENaCs, resulting in salt-induced sympathoexcitation. Three days after capsaicin treatment, an upregulation of hypothalamic α-ENaCs, without activation of mineralocorticoid receptors, was observed. We also examined expression levels of the known ENaC activator TNF-α. Hypothalamic TNF-α increased in capsaicin-treated mice, whereas intracerebroventricular infusion of the TNF-α blocker etanercept prevented capsaicin-induced upregulation of α-ENaCs. To examine brain arterial pressure (AP) sensitivity toward Na$^+$, we performed an intracerebroventricular infusion of high Na$^+$-containing (0.2 M) artificial cerebrospinal fluid. AP and heart rate were significantly increased in capsaicin-treated mice compared with control mice. CSA stimulation also caused excitatory responses with high salt intake. Compared with a regular salt diet, the high-salt diet augmented AP, heart rate, and 24-h urinary norepinephrine excretion, which is an indirect marker of sympathetic activity with mineralocorticoid receptor activation, in capsaicin-treated mice but not in ethanol-treated mice. Treatment with etanercept or the ENaC blocker benzamil prevented these salt-induced excitatory responses. In summary, we show that CSA stimulation leads to an upregulation of hypothalamic α-ENaCs mediated via an increase in TNF-α and results in increased salt sensitivity.

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MYOCARDIAL ISCHEMIA or increased left ventricular (LV) dimensions stimulate cardiac sympathetic afferent (CSA) nerve endings and sensitize the CSA reflex (31, 32, 35). This reflex is excitatory in nature and possesses positive feedback properties that result in sympathoexcitation (34). Therefore, a stimulated CSA plays an important role in the heart-brain communication for sympathoexcitation in heart failure. A recent study (30) has suggested that CSA reflex-induced sympathoexcitation promotes LV remodeling in rats with myocardial infarction. Another study (34) demonstrated that stimulation of the CSA increases hypothalamic paraventricular nucleus (PVN) neural activity, thereby eliciting reflex-mediated increases in sympathetic nerve activity. Thus, the CSA plays an important role in increasing sympathetic nerve activity in heart failure via heart-brain communication.

Salt sensitivity is known to increase in heart failure, and the activation of hypothalamic epithelial Na$^+$ channels (ENaCs) has been shown to be involved in salt-induced sympathoexcitation in heart failure (13–15). The existence of ENaCs in cardiovascular regulatory centers of the brain, including the hypothalamic PVN (1), has been clearly demonstrated to play a pivotal role particularly in salt-induced sympathoexcitation (28, 29). The mechanism underlying salt-induced sympathoexcitation has mainly been examined in salt-sensitive hypertensive rats. Activation of the hypothalamic ANG II type 1 receptor (AT1,R) is crucial to increase sympathetic activity in response to high salt loading (6, 11). However, ENaCs are known to function upstream of AT1,R activation (18, 28). Recently, we confirmed that this mechanism also exists in mice with chronic pressure overload-induced heart failure (13–15), which indicated an important role of hypothalamic ENaCs in salt-induced sympathoexcitation. However, the mechanism that connects the activation of hypothalamic ENaCs to heart failure remains unknown.

In animal models of heart failure, proinflammatory cytokines are upregulated in the hypothalamus, and their increased levels are involved in sympathoexcitation (8, 16). Furthermore, CSA stimulation is also known to modulate hypothalamic proinflammatory cytokines (5). A previous study (2) on renal ENaCs reported that proinflammatory cytokines caused enhanced Na$^+$ uptake via activation of ENaCs. Based on these findings, it was proposed that CSA stimulation, via the increased expression of proinflammatory cytokines, may result in enhanced expression of hypothalamic ENaCs.

Based on this background information, the aim of the present study was to determine the effect of CSA stimulation on levels of hypothalamic proinflammatory cytokines and to further determine the ability of these cytokines to enhance the expression of hypothalamic ENaCs, where concomitant salt loading may lead to sympathoexcitation. For this purpose, we investigated the effects of chemical CSA stimulation on the expression of hypothalamic ENaCs and on salt-induced sympathoexcitation. This was accomplished by the epicardial application of capsaicin, a chemical generally used as a tool to investigate CSA stimulation (7, 30).

**MATERIALS AND METHODS**

The study protocol was reviewed and approved by the Committee on Ethics of Animal Experiments of Kyushu University Graduate School of Medical Sciences. The study was performed in accordance with the guidelines for the care and use of laboratory animals of the Animal Care and Use Committee of Kyushu University Graduate School of Medical Sciences.
School of Medical Sciences (Fukuoka, Japan). This study was conducted according to the Guidelines for Animal Experiments of Kyushu University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

**Animals.** Ten-week-old male ICR mice (SLC, Hamamatsu, Japan) were used. In the first protocol (n = 99 mice total), mice were divided into six groups as follows: mice with epicardial application of capsaicin to stimulate CSA nerve ending receptors [transient receptor potential vanilloid (TRPV)1 receptors, n = 29; group 1] (7, 30); mice with epicardial application of ethanol as a control for epicardial application of chemicals (n = 17; group 2); mice with subcutaneous application of capsaicin or ethanol group 3 (n = 6 each; group 3); mice with epicardial application of capsaenzine (CAZ), an antagonist for TRPV1 channels, to block the effects of capsaicin (n = 12; group 4) (4, 24); mice with epicardial application of resiniferatoxin, an ultrapotent agonist for TRPV1 channels, to desensitize CSA nerve ending receptors [transient receptor potential vanilloid (TRPV)1 receptors, n = 6; group 5] (24, 27, 30); and mice with epicardial application of capsaicin and treatment with mineralocorticoid receptor (MR) blocker, TNF-α blocker, or vehicle (n = 5 for MR blocker, n = 12 for TNF-α blocker, and n = 6 for vehicle; group 6). In the second protocol (n = 76 mice total), mice with epicardial application of ethanol or capsaicin were fed on either a high-salt diet or regular salt diet between days 3 and 7, and their salt sensitivity was evaluated (n = 13 each). Mice fed with a high-salt diet also received treatment with ENaC blocker or TNF-α blocker (n = 6 each).

**Application and arterial pressure measurements.** Chemical application was allowed to occur under intraperitoneal pentobarbital sodium (25–40 mg/kg) and 1–1.5% isoflurane inhalation anesthesia and mechanical ventilation (volume-cycled rodent respirator with 1.0–1.2 ml/cycle and a respiratory rate of 110 breaths/min). An adequate depth of anesthesia was assessed by the absence of the paw withdrawal response after a noxious pinch. Afterward, the heart was exposed using a left thoracotomy. Upon removal of the pericardium, epicardial application of chemicals was performed essentially by applying a piece of filter paper (2 × 2 mm) and wrapping it in epicardial fat after it had been wetted with capsaicin (2.5 mM, 2 μl), resiniferatoxin (0.5 mM, 3 μl), or ethanol (50%, 2 μl) to the epicardial surface of the anterior wall of the LV. Subcutaneous application of capsaicin (2.5 mM, 2 μl) was also performed as a control for the epicardial application of capsaicin.

After application with chemicals, mice were monitored for arterial pressure (AP) and heart rate (HR) using a catheter [stretched polyethylene (PE)-50 tubing] inserted into the right carotid artery during chemical application. CAZ (10 mM, 3 μl) was applied 10 min before capsaicin application on the LV surface with a piece of filter paper (3 × 3 mm) to confirm TRPV1 stimulation on the LV surface. In this experiment, ethanol was used as a control for CAZ, and the AP response was evaluated after the epicardial application of capsaicin. Additionally, an undamaged CSA was evaluated by the reproducibility of capsaicin-induced AP elevations at 1-h intervals. The first epicardial application of capsaicin lasted for 30 min. Afterward, the filter paper was removed, and the surface of the LV was washed with normal saline. A second round of capsaicin treatment was carried out as described before 1 h after the first capsaicin application.

In catherized mice with epicardial application of capsaicin or ethanol, PE-50 tubing was subcutaneously tunneled from an incision made in the neck to the back of the neck, which was filled with heparinized saline after the chest was closed (15). AP and HR were measured daily (from days 0 to 3) in conscious animals after their recovery from anesthesia. After AP measurement, 100 μl blood was absorbed from the inserted catheter to measure the concentration of serum TNF-α. After 3 days of AP measurements, mice underwent echocardiography for the evaluation of LV function and were then euthanized with an overdose of pentobarbital sodium. Western blot analysis was conducted on hypothalamic tissues of mice in which catheters were not inserted to avoid the effects of catheter insertion on the expression levels of hypothalamic proteins.

Another set of mice, also treated with epicardial application of capsaicin or ethanol for 3 days, was used in high Na⁺-containing artificial cerebrospinal fluid (aCSF) intracerebroventricular infusion experiments to evaluate Na⁺ sensitivity of the brain.

In another protocol, capsaicin- or ethanol-treated animals were not euthanized on day 3 but were instead fed either a high-salt diet or a regular salt diet between days 3 and 7, and their salt sensitivity was evaluated via AP and urinary norepinephrine (uNE) excretion, which was used as a marker of sympathetic activity. Another set of mice fed a high-salt diet received intracerebroventricular infusion of TNF-α blocker or ENaC blocker. AP and uNE excretion measurements and Western blot analysis were performed on day 7. AP measurement and Western blot analysis were carried out using different animals.

**Western blot analysis.** Hypothalamic tissue was obtained from each mouse and homogenized in lysis buffer containing 40 mM HEPES, 1% Triton X-100, 10% glycerol, 1 mM sodium orthovanadate, and 1 mM PMSF. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). In total, 15 μg protein from each sample was separated on a 10% SDS-polyacrylamide gel. Proteins were subsequently transferred to polyvinylidene difluoride membranes (Immobilon-P membranes, Millipore, Billerica, MA). Membranes were incubated in an immunoreaction-enhancing solution (Can Get Signal, Toyobo, Osaka, Japan) containing rabbit IgG polyclonal antibody to the MR (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody to serum glucocorticoid-induced kinase 1 (SGK1; a marker of MR activity, 1:1,000, Abcam, Cambridge, UK), goat IgG polyclonal antibody to α-ENaC, rabbit polyclonal antibody to β-ENaC, rabbit polyclonal antibody to γ-ENaC (1:1,000, Santa Cruz Biotechnology), rabbit polyclonal antibody to TNF-α (1:1,000, Abcam), or rabbit polyclonal antibody to IL-1β (1:1,000, Abcam). Membranes were then incubated with horseradish peroxidase-conjugated horse anti-rabbit or anti-goat IgG antibody (1:10,000). GAPDH was used as an internal loading control for brain tissue. Immunoreactivity was detected using enhanced chemiluminescence autoradiography (ECL Western Blotting Detection Kit, Amersham Pharmacia Biotech, Uppsala, Sweden), and positive bands were analyzed using public domain software (ImageJ; NIH; https://rsb.info.nih.gov/nih-image/).

**Na⁺ sensitivity evaluation.** Acute responses of AP and HR to high Na⁺-containing aCSF administered by intracerebroventricular infusion were investigated. AP and HR were measured under anesthesia and mechanical ventilation via a catheter inserted in the right carotid artery. Normal Na⁺ (0.15 M)- or high Na⁺ (0.2 M)-containing aCSF was infused using a microsyringe pump at a rate of 1 μl/min for 10 min. Intracerebroventricular infusion was accomplished by incising the skin overlying the midline of the skull, and a small hole was bored using a dental drill. The hole was 0.3 mm posterior and 1 mm lateral to the bregma and 3 mm ventral to the skull surface.

To investigate 24-h uNE excretion, which is an indirect index of sympathetic activity (13–15, 33), in response to a 5-day high-salt diet, both capsaicin-treated and control mice were fed a high-salt (8%) diet or a regular salt diet between days 3 and 7 posturgery. After 5 days of the specific diet, uNE excretion was measured, and the response to the high-salt diet was evaluated in animals from each group. AP and HR of capsaicin-treated and control mice, with and without salt loading, were also measured. Under 1–2% isoflurane anesthesia, a catheter was inserted into the right carotid artery, and AP and HR were measured in each conscious mouse upon recovery from anesthesia.

**Cardiac function evaluation.** Cardiac function was evaluated echocardiographically using the AP measurements in animals on day 3, before they were euthanized. Serial M-mode echocardiography was performed on each mouse under light pentobarbital sodium anesthesia with spontaneous respiration. An echocardiography system (SSD5000, Aloka, Tokyo, Japan) with a dynamically focused...
10-MHz linear array transducer was used. M-mode tracings were recorded from the short-axis view at the level of the papillary muscle. LV end-diastolic diameter (LVDD) and LV end-systolic diameter (LVSD) were measured. The percent fractional shortening (FS) was calculated using the following equation: FS (in %) = (LVDD − LVSD)/LVDD × 100.

Treatment with the MR blocker spiranolactone. The effects of MR blockade on the expression of hypothalamic TNF-α and α-ENaCs were evaluated by starting intracerebroventricular infusion of the MR blocker spiranolactone (0.4 μg·kg⁻¹·h⁻¹, infusion rate of 0.11 μl/h) (10) 3 days before the epicardial application of capsaicin and continuing until postsurgical day 3 (i.e., 7 days in total). The skin overlying the midline of the skull was incised, and a small hole was bored as described above under isoflurane anesthesia (1–2%). Alzet brain infusion kit 3 (Durect, Cupertino, CA) connected to a dorsally implanted Alzet osmotic pump (13) was fixed to the surface of the skull with tissue adhesive. Intracerebroventricular infusion of aCSF was performed as a control for the intracerebroventricular infusion experiments.

Treatment with the TNF-α blocker etanercept. The effects of TNF-α blockade on the expression of hypothalamic TNF-α and α-ENaCs or on salt-induced sympathoexcitation were evaluated by an intracerebroventricular infusion of etanercept at 5 μg·kg⁻¹·h⁻¹ infused at a rate of 0.11 μl/h. Infusion was initiated 1 h before the epicardial application of capsaicin and was continued for 3 days, whereas protein expression was tested for 7 days when salt-induced sympathoexcitation and hypertension were tested. Intracerebroventricular infusion was performed similar to the administration of spiranolactone. A single intraperitoneal injection of etanercept (1 mg/kg) was also administered 1 h before the epicardial application of capsaicin (16). Mice pretreated with intraperitoneal injection of etanercept were also evaluated for their AP response to the epicardial application of capsaicin. This allowed the evaluation of the effects of intraperitoneal injection of etanercept on capsaicin-induced LV TRPV1 sensitization.

Treatment with the ENaC blocker benzamil. The effects of ENaC blockade on salt-induced sympathoexcitation were evaluated by intracerebroventricular infusion of benzamil, an ENaC blocker, which was used at 2.75 μg·kg⁻¹·h⁻¹ and an infusion rate of 0.11 μl/h (13). Infusion was initiated 1 h before the epicardial application of capsaicin. The intracerebroventricular infusion was performed similar to the administration of etanercept.

Measurement of serum TNF-α concentration. TNF-α concentrations were measured by ELISA (GE Healthcare UK, Buckinghamshire, UK) from cryopreserved serum samples collected from each mouse as described above.

Statistical analysis. All values are expressed as means ± SE. Two-way ANOVA was used to compare the time course of AP changes after the epicardial application of capsaicin or ethanol. One-way ANOVA was used to compare the peak responses of AP to the epicardial application of chemicals, uNE excretion, TNF-α concentrations, and protein expression levels in etanercept or benzamil intracerebroventricular infusion experiments. The Bonferroni multiple-comparison posttest was used to determine any significant differences between groups. After the equality of variance was confirmed using an F-test, an unpaired t-test was used to compare LVDDs, LVSDs, LV FS, and protein expression levels between groups. Differences were considered significant when the P value was <0.05.

RESULTS

AP, HR, and LV function after CSA stimulation. The AP of each animal (capsaicin-treated mice) increased significantly and immediately after the epicardial application of capsaicin, whereas it remained unchanged after ethanol application (ethanol-treated mice; Fig. 1, A and B). The reproducibility of capsaicin-induced AP elevation was also confirmed (Fig. 1B). Epicardial application of CAZ prevented the increase in AP induced by a subsequent application of capsaicin (Fig. 1B). However, preapplication of ethanol did not result in a significant effect on capsaicin-induced AP elevation (Fig. 1B). After recovery from anesthesia, the AP was similar between capsaicin-and ethanol-treated mice (Fig. 1A). Echocardiography re-

Fig. 1. Arterial pressure (AP) and heart rate (HR) after cardiac sympathetic afferent (CSA) stimulation. A: time course of mean AP (MAP; left) and original representative recordings of AP from mice with epicardial application of ethanol and capsaicin (right). n = 6 for each (left). *P < 0.05 vs. ethanol-treated mice. Pre, preepicardial application of the chemical; post, postepicardial (−3 min) application of the chemical; day 0, immediately after the recovery from anesthesia; days 1–3, 1–3 days after the epicardial application of chemicals. B: grouped data of peak changes in MAP after the epicardial application of each chemical. n = 6 for each. *P < 0.05 vs. ethanol-treated mice; #P < 0.05 vs. capsaicin-treated mice. CAZ, capsaizepine; CAZ + capsaicin, epicardial application of capsaicin at 10 min after epicardial application of CAZ; ethanol + capsaicin, epicardial application of capsaicin at 10 min after epicardial application of ethanol; 1st and 2nd capsaicin, epicardial application of capsaicin at 1-h intervals.
revealed that LV dimensions and LV FS did not differ between
the groups (LVDD: ethanol-treated mice 3.1 ± 0.1 mm and
capsaicin-treated mice 3.0 ± 0.1 mm; LVSD: ethanol-treated
mice 1.6 ± 0.1 mm and capsaicin-treated mice 1.5 ± 0.2 mm;
LV FS: ethanol-treated mice 48.5% ± 1.9% and capsaicin-
treated mice 47.7% ± 2.6%; n = 6 for each group).

Expression of MR, SGK1, ENaCs, and proinflammatory
cytokines. Levels of hypothalamic MR and SGK1 expression
did not differ between groups (Fig. 2). Expression levels of
hypothalamic α-ENaCs and TNF-α were higher in capsaicin-
treated mice than in control ethanol-treated mice (Fig. 2).
However, expression levels of other ENaC subunits and IL-1β
did not differ between groups (Fig. 2). Expression levels of
hypothalamic α-ENaCs and TNF-α showed a tendency to
decrease in resiniferatoxin-treated mice compared with control
mice (P = 0.06 and 0.07, respectively; Fig. 2). Furthermore,
their levels did not differ between mice with subcutaneous
application of either capsaicin or ethanol (Fig. 3A).

Enhanced Na⁺ sensitivity in capsaicin-treated mice. AP and
HR responses to intracerebroventricular infusion of normal
Na⁺-containing aCSF did not differ between ethanol- and
capsaicin-treated mice. However, these responses in capsaicin-
treated animals that received high Na⁺-containing aCSF were
significantly greater than those in ethanol-treated mice (Fig.
3B). Five-day high salt intake significantly increased AP and
HR along with an increase in uNE excretion compared with
capsaicin-treated animals consuming regular levels of salt or
ethanol-treated mice (Fig. 4, A and B). However, in ethanol-
treated control mice, high salt intake did not alter AP, HR, or
uNE excretion compared with animals with regular salt intake
(Fig. 4, A and B).

Treatment of capsaicin-treated mice with spironolactone.
Intracerebroventricular infusion of spironolactone did not de-
crease hypothalamic TNF-α and αENaC expression levels in
capsaicin-treated mice [TNF-α/GAPDH: 0.53 ± 0.05 in mice
that did not receive spironolactone (n = 6) vs. 0.50 ± 0.04 in
mice that received spironolactone (n = 5); αENaCs/GAPDH:
0.23 ± 0.04 in mice that did not receive spironolactone (n = 6) vs.
0.25 ± 0.05 in mice that received spironolactone (n = 5)].

Treatment of capsaicin-treated mice with etanercept. In
capsaicin-treated mice, both intracerebroventricular infusion
and intraperitoneal injection of etanercept decreased expres-

Fig. 2. Hypothalamic mineralocorticoid receptor (MR), serum glucocorticoid-induced kinase 1 (SGK1), epithelial Na⁺ channels (ENaCs), and proinflammatory
cytokines at 3 days after CSA stimulation. Representative Western blots demonstrate the expressions of MR and SGK1 (top), ENaCs (middle), and inflammatory
cytokines (bottom) in each group. The graph shows the means of six independent samples. Data are expressed as the relative ratio of GAPDH expression. *P <
0.05 vs. ethanol-treated mice. Resi, resiniferatoxin.

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sion levels of hypothalamic TNF-α and α-ENaCs compared with capsaicin-treated mice that did not receive etanercept (Fig. 3C). Additionally, intracerebroventricular infusion of etanercept prevented a salt-induced increase in uNE excretion, AP, and HR in capsaicin-treated mice (Fig. 4, A and B). Intracerebroventricular infusion of aCSF did not have a significant effect on the expression of these proteins in capsaicin-treated mice (Fig. 3C).

The AP response to the epicardial application of capsaicin was less in mice that received an intraperitoneal etanercept injection than in mice that did not receive it [change in mean AP: 9.8 ± 2.4 mmHg in mice that received etanercept (n = 5) vs. 19.4 ± 2.2 mmHg in mice that did not receive etanercept (n = 6)].

Treatment of capsaicin-treated mice with benzamil. Intracerebroventricular infusion of benzamil in capsaicin-treated mice prevented the salt-induced increase in uNE excretion, AP, and HR (Fig. 4, A and B).

Effects of high salt intake on hypothalamic protein expression. High salt intake in ethanol-treated mice tended to decrease the expression level of α-ENaCs (P = 0.067). However, expression levels of other proteins were not affected (Fig. 5). In contrast, high salt intake in capsaicin-treated mice increased the hypothalamic expression of α-ENaCs, SGK1, and IL-1β compared with those observed in mice with regular salt intake (Fig. 5).

Serum TNF-α concentration after CSA stimulation. Serum TNF-α concentrations did not differ between capsaicin- and
ethanol-treated mice from postsurgical days 1 to 3 (capsaicin vs. ethanol: day 1, 15.7 ± 4.4 vs. 16.2 ± 6.6 pg/ml; day 2, 19.4 ± 8.5 vs. 15.8 ± 3.5 pg/ml; and day 3, 19.3 ± 9.5 vs. 13.6 ± 10.4 pg/ml; n = 6 for each).

DISCUSSION

The present study demonstrates that chemical CSA stimulation upregulates hypothalamic α-ENaC expression and results in an augmentation of AP and HR in response to intracerebroventricular infusion of high Na⁺-containing aCSF or an increase in AP, HR, and uNE excretion in response to high salt intake. This response is induced by hypothalamic TNF-α owing to its increased expression levels in the hypothalamus upon CSA stimulation, and its blockade attenuates the increase in α-ENaC expression in the hypothalamus. These results indicate that chemical stimulation of the CSA induces salt sensitivity through TNF-α-mediated hypothalamic αENaC activation. Since CSA stimulation itself did not lead to an increase in hypothalamic MR and SGK1 expression, the increase in α-ENaC expression can be suggested to occur without MR activation. While high salt intake tended to decrease hypothalamic expression levels of α-ENaCs in ethanol-treated mice, it increased their levels in capsaicin-treated mice along with increased expression of the hypothalamic MR activity marker SGK1. High salt intake increased AP, HR, and uNE excretion only in capsaicin-treated mice. Together, these findings demonstrate that CSA-mediated heart-brain communication plays an important role in the enhancement of salt sensitivity via upregulation of hypothalamic ENaCs.

Increased CSA activity is known to be an important feature of heart failure (7, 30, 32). The stimulation of CSA results in increased neural activity in the hypothalamus (34), which, in turn, leads to excitatory sympathetic drive to the heart and deteriorating LV function (30). CSA stimulation also modulates hypothalamic proinflammatory cytokines, which have been previously reported to activate ENaCs. Salt sensitivity is increased in heart failure as well as in salt-sensitive hypertensive rats, and activation of hypothalamic ENaCs has been shown to be involved in salt-induced sympathoexcitation in chronic pressure overload-induced heart failure (13–15). Therefore, we propose the possibility that stimulation of the CSA functions as a communication pathway to enhance salt sensitivity in the case of heart failure.

Fig. 4. AP, HR, and urinary norepinephrine (uNE) excretion after salt loading. A: grouped date of MAP (left) and HR (right) in each group. n = 8. **P < 0.01 vs. ethanol-treated [both with and without high salt (HS)] and capsaicin-treated mice; *P < 0.05 vs. ethanol-treated mice [both with and without HS] and capsaicin-treated mice; #P < 0.05 vs. capsaicin-treated mice with HS. B: 24-h uNE excretion, an index of sympathetic activity, in each group. n = 8. *P < 0.05 vs. ethanol-treated mice [both with and without HS] and capsaicin-treated mice; #P < 0.05 vs. capsaicin-treated mice with HS. Ethanol HS, ethanol-treated mice with HS intake; capsaicin HS, capsaicin-treated mice with HS intake; ethanol HS + Eta, ethanol-treated mice with HS intake and intracerebroventricular infusion of etanercept; ethanol HS + Benza, ethanol-treated mice with HS intake and intracerebroventricular infusion of benzamil; capsaicin HS + Eta, capsaicin-treated mice with HS intake and intracerebroventricular infusion of etanercept; capsaicin HS + Benza, capsaicin-treated mice with HS intake and intracerebroventricular infusion of benzamil.
In the present study, we successfully demonstrated that chemical CSA stimulation increased \( \alpha \)-ENaC expression in the hypothalamus along with enhanced salt sensitivity. Despite being able to confirm that intracerebroventricular infusion of benzamil prevents salt-induced sympathoexcitation in capsaicin-treated mice, we were not able to address the precise mechanism(s) underlying salt-induced sympathoexcitation due to enhanced hypothalamic ENaCs. Hypothalamic ENaCs are well known to be involved in salt-induced sympathoexcitation in chronic pressure overload-induced heart failure models (13–15) and in salt-sensitive hypertensive rats (28, 29), in which high salt loading increases the Na\(^+\) concentration in CSF, which, in turn, leads to an increase in hypothalamic neuronal Na\(^+\) content due to activated ENaCs. These changes lead to endogenous ouabain release, and the resulting AT\(_1\)R activation is augmented (18, 28). Here, we confirmed that the hypothalamic ENaC-AT\(_1\)R pathway is necessary to induce sympathoexcitation by high salt loading in mice with chronic pressure overload-induced heart failure (15). For this reason, hypothalamic ENaCs are considered key molecules that play important roles in salt-induced sympathoexcitation. In the present study, although hypothalamic \( \alpha \)-ENaCs increased in capsaicin-treated mice, uNE excretion or AP did not differ between capsaicin-treated and control mice. In this regard, previous studies (13–15, 29) have demonstrated that AP does not increase in mice with increased hypothalamic ENaCs, but concomitant high salt loading leads to hypertension because of sympathoexcitation. In the present study, we successfully demonstrate that intracerebroventricular infusion of high Na\(^+\)-containing aCSF increases AP and HR to a greater extent in capsaicin-treated mice than in ethanol-treated mice. Additionally, high salt loading only increases AP, HR, and uNE excretion in capsaicin-treated mice. Therefore, increased hypothalamic ENaCs appear to facilitate sympathetic activation in response to salt loading.

The hypothalamic expression of MR and SGK1 was not increased by chemical CSA stimulation, suggesting that hypothalamic \( \alpha \)-ENaC expression increases without MR activation (23). In fact, intracerebroventricular infusion of spironolactone failed to prevent enhanced expression of hypothalamic \( \alpha \)-ENaCs in capsaicin-treated mice. Proinflammatory cytokines are known regulators of ENaCs (2), and
stimulation of the CSA increases their levels in the hypothalamus (5). Unfortunately, in our study, we failed to demonstrate enhanced expression of other inflammatory cytokines, such as IL-1β. Small changes in cytokine levels might not have been detected due to limitations of the Western blot technique that was used in the present study. However, we could successfully demonstrate that TNF-α blockade prevented the upregulation of hypothalamic α3-ENaCs. These results demonstrate the pivotal role of TNF-α in the enhanced expression of hypothalamic α3-ENaCs.

The MR-SGK1 pathway is an important mechanism keeping salt sensitivity in capsaicin-treated mice exposed to high salt intake. This pathway is well known to be a major ENaC activator (21), and, together, the hypothalamic MR-SGK1-ENaC pathway contributes to salt-induced sympathoexcitation (12). Previously, we (13–15) have reported that pressure overload leads to an increased extent of hypothalamic α3-ENaCs along with enhanced MR activation, which further leads to salt-induced sympathoexcitation. As described above, the hypothalamic MR pathway was not activated in capsaicin-treated mice without high salt intake. However, in capsaicin-treated mice exposed to a high-salt diet, the hypothalamic expression of SGK1 increased compared with capsaicin-treated mice with regular salt intake. In addition, hypothalamic α3-ENaC expression levels further increased in capsaicin-treated mice exposed to high salt intake, indicating that hypothalamic α3-ENaC expression remained high in capsaicin-treated mice exposed to high salt intake. Salt-induced increases in AP, HR, and uNE excretion were found only in capsaicin-treated mice, and these increases were prevented by intracerebroventricular infusion of an ENaC blocker. This indicates that high salt intake after the hypothalamic α3-ENaC increase leads to MR activation, and, as a result, the enhanced α3-ENaC expression is maintained to increase AP with sympathoexcitation.

Capsaicin is often used as a CSA stimulator (7, 30) because CSA nerve endings express a capsaicin receptor, TRPV1 (20). In the present study, we performed chemical CSA stimulation with a slightly higher dose of capsaicin than typically used in other studies since the capsaicin-containing filter paper was kept on the surface of the LV wall for several days, which raised a concern about CSA desensitization (22, 26). When capsaicin was used to ablate the sensory nerve, the dose of capsaicin was significantly higher than that in the present study (22, 26). The reproducibility of acute AP elevation in response to the epicardial application of capsaicin to negate the possibility of the sensory nerve ablation could be confirmed. Additionally, after epicardial application of the ultrapotent TRPV1 agonist and CSA desensitizer resiniferatoxin (27, 30), we found that expression levels of hypothalamic TNF-α and α3-ENaCs showed a tendency to decrease. Additionally, we could also confirm the complete inhibition of capsaicin-induced AP elevation by prior epicardial application of the TRPV1 antagonist CAZ (4, 24). Therefore, we consider that the epicardial application of capsaicin might have stimulated CSA nerve endings as a TRPV1 agonist. AP increased and peaked within 3 min after epicardial application of capsaicin. The capsaicin-induced AP elevation was transient and did not differ between capsaicin- and ethanol-treated mice. However, the possibility that a transient increase in AP influenced the mechanism of the observed enhanced salt sensitivity cannot be excluded. Salt sensitivity was evaluated 3 days after the epicardial application of chemicals to abate the effects of surgical stress on the experimentally observed data. Therefore, we are unable to propose a clear answer or directive for the necessary optimum duration of exposure to capsaicin (CSA stimulation) to enhance salt sensitivity. Further studies will be needed to gain detailed insights into these issues.

In the present study, both intracerebroventricular infusion and intraperitoneal injection of etanercept decreased TNF-α in the hypothalamus, despite intraperitoneally injected etanercept not being able to cross the blood-brain barrier (37). In a previous study (25), a systemic inflammation was reported to increase hypothalamic inflammation. Therefore, we considered the possibility that the epicardial application of capsaicin would increase systemic TNF-α, which would be expected to increase hypothalamic TNF-α levels. Contrary to this, the serum TNF-α concentration did not differ between capsaicin- and ethanol-treated mice, and the subcutaneous application of capsaicin did not change hypothalamic TNF-α expression levels. TNF-α has been reported to sensitize TRPV1 channels to capsaicin (17), and TRPV1 activation is known to increase TNF receptors (19). Based on these results, we considered the possibility that pretreatment with intraperitoneal etanercept might attenuate the impact of the epicardial application of capsaicin on LV TRPV1 channels. Therefore, we examined the AP response to the epicardial application of capsaicin after pretreatment with intraperitoneal etanercept and found that the capsaicin-induced AP elevation was attenuated by such pretreatment. These results suggest that intraperitoneally injected etanercept might decrease TNF-α levels in the hypothalamus because of an attenuated impact of capsaicin on LV TRPV1 channels. Furthermore, we also found that epicardially applied resiniferatoxin-mediated CSA desensitization tended to decrease hypothalamic TNF-α expression levels. Intracerebroventricular infusion of etanercept could successfully decrease the levels of hypothalamic TNF-α and α3-ENaCs. Therefore, 

![Fig. 6. Hypothetical schema of the link between the heart and brain in the enhanced salt sensitivity for sympathetic activity. CSAR, CSA reflex.](http://ajp.heart physiology.org)
we believe that the CSA has a pivotal role in increasing hypothalamic TNF-α levels.

In the present study, drugs were administered to mice using intracerebroventricular infusion methods. Intracerebroventricularly infused drugs are known to spread widely in the brain, and because of this we could not come to the clear conclusion that the hypothalamus is the sole seat that mediates the effects of administered drugs. The hypothalamus is known to contribute to enhanced salt sensitivity (28, 29), and, in this regard, we could demonstrate the attenuation in salt sensitivity with decreased expression of proteins in the hypothalamus. Although the hypothalamus is a broad region divided into several specific nuclei according to their functions, we examined protein expression levels using whole tissues of the hypothalamus in the present study. The hypothalamic PVN is, however, known as a center of autonomic function and is involved in the regulation of sympathetic activity (3). Several studies (9, 34, 36) have demonstrated that the hypothalamic PVN plays an important role in the CSA reflex as its central pathway. In fact, CSA activation is reported to increase neural activity in the PVN of the hypothalamus (34). Therefore, we propose that the hypothalamic PVN contributes to enhanced salt sensitivity due to CSA stimulation and that intracerebroventricularly infused drugs work particularly in the hypothalamic PVN of the hypothalamus to attenuate salt sensitivity.

In summary, in the present study, we demonstrate, for the first time, that CSA stimulation induces TNF-α-mediated salt sensitivity through activation of hypothalamic ENaCs in the brain. In this regard, the importance of the link between heart-brain communication was established in the present study (Fig. 6).

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: K.I. conception and design of research; K.I. performed experiments; K.I. analyzed data; K.I., Y.H., and K.S. interpreted results of experiments; K.I. prepared figures; K.I. drafted manuscript; K.I. and Y.H. edited and revised manuscript; K.I., Y.H., and K.S. approved final version of manuscript.

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