Central SDF-1/CXCL12 expression and its cardiovascular and sympathetic effects: the role of angiotensin II, TNF-α, and MAP kinase signaling

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Wei SG, Zhang ZH, Yu Y, Felder RB. Central SDF-1/CXCL12 expression and its cardiovascular and sympathetic effects: the role of angiotensin II, TNF-α, and MAP kinase signaling. Am J Physiol Heart Circ Physiol 307: H1643–H1654, 2014. First published September 26, 2014; doi:10.1152/ajpheart.00432.2014.—The chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) and its receptors are expressed by neurons and glial cells in cardiovascular autonomic regions of the brain, including the hypothalamic paraventricular nucleus (PVN), and contribute to neurohumoral excitation in rats with ischemia-induced heart failure. The present study examined factors regulating the expression of SDF-1 in the PVN and mechanisms mediating its sympato-excitatory effects. In urethane anesthetized rats, a 4-h intracerebroventricular (ICV) infusion of angiotensin II (ANG II) or tumor necrosis factor-α (TNF-α) in doses that increase mean blood pressure (MBP) and sympathetic drive increased the expression of SDF-1 in PVN. ICV administration of SDF-1 increased the phosphorylation of p44/42 mitogen-activated protein kinase (MAPK), JNK, and p38 MAPK in PVN, along with MBP, heart rate (HR), and renal sympathetic nerve activity (RSNA), but did not affect total p44/42 MAPK, JNK, and p38 MAPK levels. ICV pretreatment with the selective p44/42 MAPK inhibitor PD98059 prevented the SDF-1-induced increases in MBP, HR, and RSNA; ICV pretreatment with the selective JNK and p38 MAPK inhibitors attenuated but did not block these SDF-1-induced excitatory responses. ICV PD98059 also prevented the sympato-excitatory response to bilateral PVN microinjections of SDF-1. ICV pretreatment with SDF-1 short-hairpin RNA significantly reduced ANG II- and TNF-α-induced phosphorylation of p44/42 MAPK in PVN. These findings identify TNF-α and ANG II as drivers of SDF-1 expression in PVN and suggest that the full expression of their cardiovascular and sympathetic effects depends upon SDF-1-mediated activation of p44/42 MAPK signaling.

IN A VARIETY OF CENTRAL NERVOUS SYSTEM disorders, chemokines are considered to be important mediators and modulators of the central inflammatory response (30, 35, 37, 49). The contribution of chemokines to the central inflammatory state that promotes neurohumoral excitation in experimental models of hypertension and heart failure is largely unexplored. We recently reported that the chemokine stromal cell-derived factor-1/CXCL12 (SDF-1) is upregulated in key cardiovascular/autonomic regions of the brain - namely, the hypothalamic paraventricular nucleus (PVN), the supraoptic nucleus, and the subfornical organ - in rats with heart failure and that SDF-1 contributes significantly to neurohumoral excitation in that condition (59). However, the factors that upregulate brain SDF-1 expression in heart failure and the intracellular mechanisms that mediate its effects on neurohumoral regulation are unknown.

Brain renin-angiotensin system activity (16, 68) and the pro-inflammatory cytokines (17, 39) are upregulated in cardiovascular/autonomic regions of the brain in heart failure and hypertension, and contribute to the excitatory neurochemical milieu that drives sympathetic nerve activity. Angiotensin II (ANG II) and the prototypical pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) can both stimulate neuronal and glial elements that express SDF-1 (6). Thus we tested the hypothesis that ANG II and TNF-α upregulate SDF-1 expression in the PVN. In addition, because we have found that mitogen-activated protein kinase (MAPK) signaling is required for sympathetic activation in heart failure (55) and hypertension (63), we tested the hypothesis that MAPK signaling mediates the sympatho-excitatory effects of SDF-1. Findings that ANG II and TNF-α induce SDF-1 expression in the PVN, and that MAPK signaling mediates SDF-1-induced sympathetic excitation, would suggest a previously unrecognized central mechanism contributing to sympathetic drive in heart failure.

This study focused specifically on neurochemical events in the PVN, where neuroinflammation has been shown to play an important role in sympathetic activation (27, 65), and on the inflammatory chemokine SDF-1, which our previous study demonstrated to be increased in the PVN of heart failure rats.

METHODS

Animals. Adult male Sprague-Dawley rats, weighing 300–350 g, were purchased from Harlan Sprague Dawley (Indianapolis, IN). The animals were housed in temperature-controlled (23 ± 2°C) rooms in University of Iowa Animal Care Facility and fed rat chow ad libitum. All experimental procedures were reviewed and approved by The University of Iowa Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Acute electrophysiological and hemodynamic recording. The surgical preparation for electrophysiological and hemodynamic recording has been detailed in our previous publications (53, 55). Briefly, rats were anesthetized with urethane (1.5 g/kg ip), supplemented as needed (0.1 g/kg iv). Blood pressure (BP) was measured via a catheter implanted in the left femoral artery. Body temperature was maintained at 37 ± 1°C with a heating pad and heat lamp. A renal nerve was exposed via a left flank incision and was dissected free from surrounding tissue. The renal nerve was placed on bipolar silver wire recording electrodes to record renal sympathetic nerve activity (RSNA). The incisions were closed after nerve and electrodes were stabilized with Kwik-Cast silicon sealant (WPI, Sarasota, FL). The recording session began when baseline RSNA and BP were stable, at least 60 min following completion of the surgical preparations.

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For intracerebroventricular (ICV) drug administration, animals were fixed in a stereotaxic frame and implanted with a 29-gauge stainless steel guide cannula with the tip of the cannula placed 2 mm above the left lateral cerebral ventricle using the following coordinates: anteroposterior, −1.0 mm; dorsoventral, −2.5 mm; and mediolateral, −1.5 mm, with bregma as a reference. A 33-gauge injection cannula connected to 10 μl micro-syringe was inserted into the guide cannula and was extended 2 mm past the tip of the guide cannula. The injections were performed with a micropump (Harvard Apparatus, Holliston, MA).

The method for bilateral PVN microinjection has been described in detail previously (59). Briefly, one 30-gauge guide cannula was placed 1.8 mm posterior to bregma, 0.4 mm left of midline for PVN microinjection. Another 30-gauge guide cannula was placed 1.4 mm right of midline but angled 10° toward midline for right PVN microinjection. Both cannula tips were advanced to a final position 5.8 mm ventral to the cranial surface. The 35-gauge injection cannulas connected with 1-μl Hamilton micorsyringes were inserted into the guide cannulas and extended 2 mm beyond the tip of the guide cannula for bilateral simultaneous PVN microinjection (0.2 μl over 10 s). The microinjection sites were marked by injecting 2% Pontamine sky blue (100 nl) at the same locations at the end of each experiment.

Data were acquired with a Cambridge Electronics Design laboratory interface (CED, model 1401; Cambridge, UK) connected to a personal computer. RSNA was initially processed with a Paynter filter (20-ms time constant; BAK Electronics, Germantown, MD) to rectify and integrate the raw multifiber signal. The BP signal was passed to the CED 1401 via a Gould TA240S chart recorder (Gould Instruments, Valley View, OH). Heart rate (HR) was derived from the frequency of the BP pulses. Digitized data were stored for subsequent offline analysis with Spike2 software (CED).

**ICV administration of lentiviral particles.** Rats were anesthetized with ketamine-xylazine (90 mg/kg + 10 mg/kg ip) and placed in a stereotaxic headholder. Under sterile conditions, the skull was exposed and a small hole was drilled over the left lateral ventricle. A 33-gauge injection cannula connected with a 1-μl Hamilton microsyringe was lowered to the lateral ventricle using the following coordinates: anteroposterior, −1.0 mm; dorsoventral, −4.5 mm; and mediolateral, −1.5 mm. SDF-1 short-hairpin RNA (shRNA) lentiviral particles or scrambled shRNA lentiviral particles were injected (10 μl, 0.1 μg/μl over 5 min). After 10 min, the cannula was removed, the incision was closed, and the rats were returned to their cages to recover.

**Experimental protocols.** Four protocols were used.

**PROTOCOL 1.** Urethane-anesthetized rats received a 4-h ICV infusion of ANG II (50 ng/h), TNF-α (50 ng/h), or vehicle (Veh) and then were euthanized while still under anesthesia to collect brain tissue to determine the expression of SDF-1 in the PVN, measured by Western blot analysis and immunofluorescence.

**PROTOCOL 2.** Urethane-anesthetized rats received a 4-h ICV infusion of SDF-1 (50 ng/h) or Veh and then were euthanized while still under anesthesia to collect brain tissue to determine the expression of activated (phosphorylated, p-) form of the three major terminal effectors of MAPK kinases of the MAPK family - p44/42 MAPK (also known as ERK 1/2), p38 MAPK, and the stress-activated protein kinase/JNK - in the PVN, as determined by Western blot analysis and immunofluorescent analysis.

**PROTOCOL 3.** Urethane-anesthetized rats underwent electrophysiological and hemodynamic recording studies to determine the role of p-p44/42 MAPK, p-p38 MAPK, and p-JNK in mediating the cardiovascular and sympathetic responses to centrally administered SDF-1.

Some rats received ICV injections of SDF-1 (100 ng) preceded by ICV injection of Veh, the p44/42 MAPK inhibitor PD98059 (1 mg), the p38 MAPK inhibitor SB203580 (1 mg), or the JNK inhibitor SP600125 (1 mg).

Other rats received bilateral PVN microinjections of SDF-1 (10 ng each side) preceded by bilateral PVN microinjections of Veh or PD98059 (0.1 mg each side) to further examine the role of p-p44/42 MAPK signaling in PVN.

**PROTOCOL 4.** Rats underwent sterile surgery under ketamine-xylazine anesthesia to receive an ICV injection of SDF-1 shRNA lentiviral particles or a scrambled shRNA. One week later, under urethane anesthesia, they underwent a 4-h ICV infusion of ANG II (50 ng/h), TNF-α (50 ng/h), or Veh.

Some rats were then transcardially perfused with 4% paraformaldehyde, and the brains were collected for immunofluorescent studies to determine the effect of ICV SDF-1 shRNA on SDF-1 expression in the PVN and other cardiovascular regions and the effect of reducing SDF-1 on p-p44/42 MAPK expression in the PVN.

Other rats were euthanized, and brain tissue was collected to determine the effect of the SDF-1 shRNA on SDF-1 mRNA, measured by real-time PCR (RT-PCR).

**Drug administration.** For Western and immunofluorescent studies, SDF-1/CXCL12 (Catalog No. 350-NS-050/CF; R&D Systems, Minneapolis, MN), ANG II (Sigma, St. Louis, MO), and TNF-α (Fitzgerald, Acton, MA) were diluted in artificial cerebrospinal fluid (aCSF). aCSF served as the control. The ICV doses of SDF-1 shRNA lentiviral particles (Catalog No. sc-39367-V; Santa Cruz, CA) and scrambled shRNA lentiviral particles (Catalog No. sc-108080; Santa Cruz, CA) were according to our previous study (59) and the manufacturer’s instructions.

For the electrophysiological studies, SDF-1 was dissolved in aCSF. Doses of SDF-1 were the same as those used in a previous publication (59).

The selective MAPK inhibitors PD98059, SP600125, and SB203580 were obtained from Tocris (Ellisville, MO). These inhibitors were dissolved in DMSO first and then diluted in aCSF to make a 5% final DMSO concentration. The vehicle (Veh) was aCSF containing 5% DMSO. ICV doses of the inhibitors were substantially larger than those required to reduce MAPK activity in rats with heart failure (55, 56).

**Western blot.** Rats anesthetized with urethane (1.5 g/kg ip) were decapitated to obtain brain tissue for Western blot. The brains were immediately removed, frozen in liquid nitrogen, and stored at −80°C for subsequent use. The frozen brains were cut into 300-μm coronal sections. Target tissues were obtained using a punch device (inner diameter, 1.5 mm; Stoelting, Wood Dale, IL) and were homogenized in cell lysis buffer consisting of 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1 μg/ml leupeptin (Cell Signaling Technology, Beverly, MA) containing the protease inhibitor (Complete, Mini, EDTA-free, Catalog No. 11 836 170 001; Roche Diagnostics) to extract protein for Western assay.

Phosphorylated p44/42 MAPK, p38 MAPK, and JNK were detected using antibodies (1:500; Cell Signaling Technology, Beverly, MA) to p-p44/42 MAPK (Catalog No. 4377), p-p38 MAPK (Catalog No. 9215), and p-JNK (Catalog No. 9251), respectively. Total p44/42 MAPK, p38 MAPK, and JNK were detected using rabbit monoclonal antibodies (1:1,000; Cell Signaling Technology) to p44/42 MAPK (Catalog No. 4695), p38 MAPK (Catalog No. 8690), and JNK (Catalog No. 9258), respectively. SDF-1 was detected using polyclonal antibodies against SDF-1 (SC-6193, 1:300; Santa Cruz, CA). The second antibodies were goat anti-rabbit IgG-HRP (SC-2054, 1:5,000; Santa Cruz) or rabbit anti-goat IgG-HRP (sc-2922, 1:5,000; Santa Cruz). Immunoblots were visualized with an enhanced chemiluminescence reagent. Band intensities were quantified with NIH ImageJ software. The content of p-p44/42, p-JNK, p-p38, and SDF-1 were normalized to total β-actin.

**RT-PCR.** Urethane anesthetized rats (1.5 g/kg ip) were decapitated, and the brains were immediately removed and frozen in liquid nitrogen. The frozen brain was cut into 300-μm coronal sections, and...
the PVN was punched using a 15-gauge needle (inner diameter 1.5 mm). SDF-1 mRNA levels in PVN were measured with RT-PCR following reverse transcription of total RNA. The sequences for primers and probe used were as follows: sense primer, 5'-ATG-AAC-GCC-AAG-GTC-GTG-GTC-3'; antisense primer, 5'-TGG-CTG-TTG-TGC-TTA-CITT-GTT-T-3'. TaqMan primer and probe for rat GAPDH were purchased from Applied Biosystems (Foster City, CA). RT-PCR was performed using the ABI prism 7700 Sequence Detection System (Applied Biosystems). GAPDH mRNA was quantified as an internal control for each sample. The value for each sample was normalized to GAPDH and expressed as a fold difference relative to the control.

**Immunofluorescence.** Rats anesthetized with urethane (1.5 g/kg ip) were transcardially perfused with 4% paraformaldehyde. Brains were removed and embedded with OCT and rapidly frozen in acetone chilled by dry ice. Coronal forebrain sections (16 μm) of target tissues were made using a cryostat and stored at −80°C for subsequent staining.

The sections were incubated with primary rabbit monoclonal antibodies (1:100; Cell Signaling Technology) to p-p44/42 MAPK (Catalog No. 4376), p-p38 MAPK (Catalog No. 4511), or p-JNK (Catalog No. 4668), and/or the mouse monoclonal antibody to neuronal marker NeuN (MAB377, 1:300; Millipore), followed by secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (A-11070, 1:200; Invitrogen) and Alexa Fluor 568 goat anti-mouse IgG (A-11003, 1:200; Invitrogen). The same antibodies used in Western analysis were used to detect SDF-1 (SC-6193, 1:100) by immunofluorescence. The secondary antibodies were Alexa Fluor 488 donkey anti-goat IgG (A-10037, 1:200; Invitrogen) and Alexa Fluor 568 donkey anti-rabbit IgG (A-11070, 1:200; Invitrogen). The specificity of the antibody to detect the expression of SDF-1 in rat brain tissue by immunofluorescence has been tested previously (6). For immunofluorescent analysis of phosphorylated MAPK and SDF-1, the fluorescent intensity was measured in the four different subdivisions of PVN: the dorsal parvocellular (PVN-dp), the medial parvocellular (PVN-mp), ventrolateral parvocellular (PVN-vlp), and the magnocellular subdivision (PVN-pm). The fluorescent intensity was averaged over three representative transverse sections (100 μm²) in each animal and expressed as arbitrary units per square micrometer.

**Statistical analysis.** Electrophysiological and hemodynamic data were analyzed with Spike2 software. Mean blood pressure (MBP; in mmHg), HR (in beats/min), and RSNA as windowed multifiber action potential discharge (spikes/s) and as rectified and integrated voltage (in mV) were averaged over 5-min intervals at baseline and during each intervention. The maximum MBP, HR, and RSNA response to each intervention was compared with the immediately preceding baseline values. For RSNA, the percent change in the integrated voltage signal was used for data analysis. All values are expressed as the means ± SE. The significance of differences among groups was analyzed by two-way repeated-measure ANOVA followed by post hoc Fisher’s least significant difference test.

**RESULTS**

**Effects of ICV ANG II and TNF-α on the expression of SDF-1 in the PVN.** A 4-h ICV infusion of ANG II or TNF-α significantly increased the expression of SDF-1 in the PVN (Fig. 1). Western blot analysis demonstrated a marked increase

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**Fig. 1.** Immunofluorescent (A and B) and Western blot (C) data showing the expression of stromal cell-derived factor (SDF)-1 in the hypothalamic paraventricular nucleus (PVN) of rats treated with a 4-h intracerebroventricular (ICV) infusion of ANG II, TNF-α, or vehicle (Veh). Approximate subdivisions of the PVN are indicated in A. middle: dp, dorsal parvocellular; mp, medial parvocellular; vlp, ventrolateral parvocellular; and pm, posterior magnocellular. Values are expressed as means ± SE (n = 6 for each group). The SDF-1 level in Western analysis was normalized to β-actin. *P < 0.05 compared with Veh. Scale Bar: 200 μm. AU, arbitrary units.
in SDF-1 protein in the PVN (P < 0.05) in ANG II- or TNF-α-treated rats compared with Veh-treated rats. Immunofluorescent confocal images revealed increased SDF-1 expression in dorsal parvocellular, medial parvocellular, ventrolateral parvocellular, and magnocellular regions of PVN, the four commonly recognized subdivisions.

**Effects of ICV SDF-1 on MAPK expression in PVN.** A 4-h ICV infusion of SDF-1 significantly increased phosphorylated MAPK expression in PVN. Immunofluorescent confocal images (A) and Western blots (C) show increased phosphorylated p44/42 MAPK, p38 MAPK, and JNK in the PVN of rats treated with a 4-h ICV infusion of SDF-1 or Veh. Subdivisions of PVN, as illustrated in Fig. 1, are dp, mp, vlp, and pm. Values are expressed as means ± SE (n = 6 or 7 for each group). The levels of phosphorylated and total p44/42 MAPK, p38 MAPK, and JNK protein in Western analysis were first normalized to β-actin and then displayed as a ratio of phosphorylated to total p44/42 MAPK, p38 MAPK, and JNK. *P < 0.05 compared with Veh. Scale Bar: 200 μm.

**Fig. 2.** Immunofluorescent (A and B) and Western blot (C) data showing the expression of total and phosphorylated (p-) p44/42 MAPK, p38 MAPK, and JNK in the PVN of rats treated with a 4-h ICV infusion of SDF-1 or Veh. Subdivisions of PVN, as illustrated in Fig. 1, are dp, mp, vlp, and pm. Values are expressed as means ± SE (n = 6 or 7 for each group). The levels of phosphorylated and total p44/42 MAPK, p38 MAPK, and JNK protein in Western analysis were first normalized to β-actin and then displayed as a ratio of phosphorylated to total p44/42 MAPK, p38 MAPK, and JNK. *P < 0.05 compared with Veh. Scale Bar: 200 μm.
Fig. 3. Representative tracings (A–D) and grouped data (E–G) showing the effects of an ICV injection of SDF-1 (100 ng) on blood pressure (BP; in mmHg), heart rate [HR; in beats/min (bpm)], and renal sympathetic nerve activity (RSNA), windowed (in spikes/s) and rectified and integrated (in mV), in rats pretreated with ICV Veh (A), PD98059 (B), SB203580 (C), or SP600125 (D). Arrows indicate the initiation of the injections. Scale Bar: 20 min. MBP, mean blood pressure; ΔRSNA (in %), percent change from baseline in integrated RSNA. All values are expressed as the means ± SE. *P < 0.05 compared with baseline; †P < 0.05 compared with Veh-pretreated animals.
MAPK expression in the PVN (Fig. 2). Immunofluorescent reactivity for p-p44/42 MAPK, p-p38 MAPK, and p-JNK increased significantly in all four subdivisions of the PVN. The increase in p-p44/42 MAPK was largely confined to the PVN; immunofluorescence for p-p38 MAPK and p-JNK increased in the PVN and the immediately surrounding hypothalamic tissues. Western blot analysis demonstrated substantially higher (P < 0.05) levels of p-p44/42 MAPK, p-p38 MAPK, and p-JNK in the PVN of SDF-1-treated rats than of Veh-treated rats, but no significant change in total p44/42 MAPK, p38 MAPK, or JNK.

MAPK-mediated effects of SDF-1 on hemodynamics and RSNA. ICV injection of SDF-1 induced significant increases in MBP, HR, and RSNA (Fig. 3, A and E–G). These excitatory responses to SDF-1 began within 10 min of the ICV injection. The maximum responses of MBP (33.9 ± 3.7 mmHg), HR (95.7 ± 12.5 beats/min), and RSNA (105.9 ± 10.2 %change) occurred 3 to 4 h after ICV administration of SDF-1, and above baseline levels persisted for 5–8 h and even longer in most cases.

ICV pretreatment with the p44/42 MAPK inhibitor PD98059 prevented the SDF-1-evoked increases in MBP, HR, and RSNA (Fig. 3, B and E–G). ICV pretreatment with the p38 MAPK inhibitor SB203580 (Fig. 3, C and E–G) and the JNK inhibitor SP600125 (Fig. 3, D and E–G) significantly attenuated but did not block the excitatory responses to ICV SDF-1: MBP (19.4 ± 3.5 and 17.1 ± 3.9 mmHg, respectively), HR (54.1 ± 12.8 and 46.6 ± 13.1 beats/min, respectively), and RSNA (52.2 ± 10.5 and 58.6 ± 9.4 %change, respectively). ICV injections of an equal volume of vehicle (aCSF with 5% DMSO) had no noticeable effect on baseline MBP (99.5 ± 3.0 mmHg), HR (320 ± 14 beats/min), or integrated RSNA (10.6 ± 3.3 mV).

Bilateral PVN microinjections of SDF-1 elicited similar excitatory hemodynamic and sympathetic responses (Fig. 4, A and C–E). Pretreatment with bilateral PVN microinjections of the p44/42 MAPK inhibitor PD98059 prevented these responses (Fig. 4, B and C–E): peak MBP (from 20.4 ± 3.3 to 6.2 ± 3.0 mmHg), HR (from 64.6 ± 11.7 to 15.9 ± 9.5 beats/min), and RSNA (from 75.8 ± 10.5 to 14.6 ± 8.6% change). Bilateral PVN microinjection of vehicle had no effect on baseline MAP, HR, and RSNA. Histological evaluation of brain sections following these experiments confirmed that the marked microinjection sites were located within the confines of the PVN (33).

SDF-1-mediated effects of ANG II and TNF-α on p-p44/42 MAPK expression in PVN. In rats pretreated 1 wk earlier with an ICV injection of scrambled shRNA lentiviral particles, a 4-h ICV infusion of ANG II or TNF-α induced a substantial increase in p-p44/42 MAPK immunofluorescence in all four major subdivisions of the PVN (Fig. 5). These increases in p-p44/42 immunofluorescence were significantly attenuated in the PVN of rats that had been pretreated with SDF-1 shRNA lentiviral particles (Fig. 5).

Additional studies were performed to assess the effectiveness of the SDF-1 shRNA lentiviral particles. Rats pretreated with ICV SDF-1 shRNA had significantly lower PVN levels of SDF-1 mRNA in response to TNF-α and ANG II than rats pretreated with the scrambled control shRNA (Fig. 6). The effect of ICV SDF-1 shRNA lentiviral particles on SDF-1 immunoreactivity, examined after the ICV TNF-α infusion,
was not confined to PVN. Immunoreactivity for SDF-1 was also reduced in the subfornical organ and supraoptic nucleus (Fig. 7).

**DISCUSSION**

We previously reported increased expression of the chemokine SDF-1/CXCL12 in the PVN and the subfornical organ of heart failure rats (59). In that study, microinjection of SDF-1 into the PVN elicited exaggerated increases in MBP, HR, and RSNA in heart failure rats, and ICV administration of SDF-1 shRNA lentiviral particles (B). Fluorescent intensity data are shown in C. Scale bar: 200 μm. Immunofluorescent intensity (in AU) is expressed as means ± SE (n = 6 for each group). Subdivisions of PVN, as illustrated in Fig. 1, are dp, mp, vlp, and pm. *P < 0.05 compared with ICV scrambled control shRNA + Veh; †P < 0.05, ICV SDF-1 shRNA vs. ICV scrambled control shRNA.

Fig. 5. Immunofluorescent confocal images showing the expression of phosphorylated p44/42 MAPK (p-p44/42) in the PVN of rats treated with a 4-h ICV infusion of Veh, ANG II, or TNF-α in rats pretreated 1 wk earlier with ICV injection of a scrambled control short-hairpin RNA (A; shRNA) or SDF-1 shRNA lentiviral particles (B). Fluorescent intensity data are shown in C. Scale bar: 200 μm. Immunofluorescent intensity (in AU) is expressed as means ± SE (n = 6 for each group). Subdivisions of PVN, as illustrated in Fig. 1, are dp, mp, vlp, and pm. *P < 0.05 compared with ICV scrambled control shRNA + Veh; †P < 0.05, ICV SDF-1 shRNA vs. ICV scrambled control shRNA.

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The present study in normal rats sought to define factors that might upregulate SDF-1 expression in the PVN and mediate its cardiovascular and sympathetic effects. The major findings are 1) ICV ANG II and TNF-α increase SDF-1 expression in the PVN; 2) ICV SDF-1 activates MAPK signaling cascades in the PVN; 3) MAPK signaling mediates the hemodynamic and sympathetic responses to ICV SDF-1, with p44/42 MAPK having the predominant influence; and 4) ANG II and TNF-α-driven activation of p44/42 MAPK signaling is at least partially mediated by SDF-1.
Astrocytes, microglia, and neurons all express SDF-1 (6, 59) and the two receptors that mediate its effects: CXCR4 and CXCR7 (3, 23, 29, 31). Many effects of SDF-1 are blocked by the selective CXCR4 receptor antagonist AMD 3100, suggesting that CXCR4 is the primary SDF-1 receptor. The CXCR7 receptor is less well understood but may modulate the actions of SDF-1 on CXCR4 (34, 35, 51). In a recent study of the distribution of CXCR4 and CXCR7 in differentiated neurons (41), CXCR4 was found to be expressed both at the cell membrane and in the cytoplasm, and was considered the main mediator of the effects of SDF-1. CXCR7 was mostly confined to the cytoplasm, where it likely interacts with CXCR4 to modulate its intracellular functions and perhaps its expression at the membrane (41). In rat neurons, SDF-1-induced phosphorylation of p44/42 MAPK was unaffected by ligands that activated CXCR7 receptors and eliminated their ability to respond to SDF-1 (41).

The distribution of CXCR4 in the brain closely resembles the distribution of SDF-1, including its expression in PVN. In rats with ischemia-induced heart failure, SDF-1 expression increases diffusely throughout the PVN (59), potentially affecting both sympathetic and neuroendocrine neurons. In the present study, acute ICV infusions of ANG II and TNF-α, two major determinants of the neurohumoral excitation in heart failure, caused a similar diffuse upregulation of SDF-1 expression in the PVN.

ANG II and TNF-α are known to upregulate SDF-1 expression in peripheral tissues (10, 13, 18), and TNF-α can increase the SDF-1 expression in cultured astrocytes and endothelial cells (45). However, to our knowledge this is the first report to demonstrate that ANG II and TNF-α upregulate SDF-1 expression in an important cardiovascular regulatory center of the brain and that SDF-1 contributes to their sympatho-excitatory effects. The molecular mechanisms responsible for ANG II and TNF-α upregulation of SDF-1 are currently unknown. However, ANG II and TNF-α both induce oxidative stress (21, 69) and endoplasmic reticulum stress (12, 61), processes that stimulate MAPK pathways and NF-κB signaling (24, 40, 46). These molecular mechanisms have been implicated in the upregulation of other chemokines [e.g., CCL2 monocyte chemoattractant protein-1 (MCP-1) and CCL7/MCP-7 in cultured rat astrocytes] (48) and may well contribute to ANG II and TNF-α upregulation of SDF-1 (and potentially other chemokines) in cardiovascular regions of the brain.

In the present study, we identify MAPK signaling as an intracellular mechanism that mediates the sympatho-excitatory effects of SDF-1. This finding is not without precedent. In vitro studies have shown that the effect of SDF-1 to stimulate the release of the excitatory fast neurotransmitter L-glutamate and TNF-α from astrocytes is blocked by selective inhibitors of p44/42 MAPK signaling (8). L-glutamate is known to induce MAPK signaling (25, 32), and MAPK signaling has been implicated in the activation of vasopressinergic (52) and corticotropin releasing hormone (42) neurons in the PVN. Our recent work has established an important role for p44/42 MAPK signaling in the neurohumoral excitation associated with heart failure (55, 56) and hypertension (63).

The present study revealed that ICV ANG II and ICV TNF-α upregulate the expression of SDF-1 in the PVN and that ICV SDF-1 upregulates the expression of p-p44/42 MAPK, p-JNK, and p-p38 MAPK in the PVN. SDF-1-induced p-p44/42 MAPK was confined to the PVN, whereas p-p38 MAPK and p-JNK were expressed in similar densities in the immediately surrounding hypothalamic tissues (Fig. 2). The expression of p-p44/42 localized within the borders of the PVN was significantly reduced by pretreatment with the SDF-1 shRNA (Fig. 5). These results suggest that ANG II- and TNF-α-induced SDF-1 activity contributes to their upregulation of MAPK activity in PVN and the resulting sympatho-excitatory activation, with p-p44/42 MAPK playing a major role. Consistent with that hypothesis, the pressor response induced by the ICV SDF-1 infusion was completely blocked by pretreatment with a selective p44/42 MAPK inhibitor, whereas the selective JNK and p38 MAPK inhibitors had only moderate although significant effects. More direct evidence was provided by PVN microinjections of the p44/42 MAPK inhibitor, which blocked the sympatho-excitatory response to the subsequent PVN microinjection of SDF-1.

The question of how brain MAPK signaling leads to neurohumoral excitation remains unanswered. The phosphorylated MAPKs - p-p44/42 MAPK, p-JNK, and p-p38 MAPK - have both nuclear and cytoplasmic effects (50) and may have both long- and short-term effects on the excitability of PVN neurons. Acting upon nuclear transcription factors, including NF-κB, activator protein 1, and cyclic adenosine monophosphate response element binding protein, they may increase the production of components of the brain renin-angiotensin system (angiotensinogen, angiotensin converting enzyme, angiotensin II type 1 receptors) and inflammatory mediators (TNF-α, interleukin-1β, cyclooxygenase-2), potentially exacerbating the excitatory neurochemical milieu in chronic settings like heart failure and hypertension. All three MAPKs have been implicated in ANG II upregulation of the angiotensin II type 1 receptor (54, 56), and p44/42 MAPK and p38 MAPK have been implicated in upregulation of angiotensin converting enzyme (60).
In addition, MAPK signaling may modulate the transient outward potassium current (20) that normally restrains neuronal excitability in cardiovascular related central nuclei (2, 7, 11, 14, 19, 28, 43, 44). The duration of the delay in neuronal excitation following hyperpolarization (i.e., induced by gamma aminobutyric acid [GABA]) is largely determined by the expression of the potassium channel subunits Kv4.2 and Kv4.3 (28). ANG II-induced downregulation of the Kv4.3 subunit in rostral ventrolateral medulla, attributed to p38 MAPK signaling, has been associated with increased sympathetic activity in 

Fig. 7. Confocal images showing SDF-1 immunofluorescence in the PVN (A and B), subfornical organ (SFO; C and D), and supraoptic nucleus (SON; E and F) following a 4-h ICV infusion of TNF-α in rats treated 1 wk earlier with ICV scrambled control shRNA (A, C, and E) or ICV SDF-1 shRNA lentiviral particles (B, D, and F). Scale bars: 200 μm. Grouped data show SDF-1 fluorescent intensity for ICV TNF-α-induced SDF-1 in PVN (G), SFO, and SON (H) in rats treated 1 wk earlier with ICV SDF-1 shRNA lentiviral particles or ICV scrambled control shRNA. Subdivisions of PVN, as illustrated in Fig. 1, are dp, mp, vlp, and pm. Immunofluorescent intensity (in AU) is expressed as means ± SE (n = 4 for each group). *P < 0.05 vs. ICV control shRNA + TNF-α.
heart failure (19, 43). A more immediate effect of MAPK signaling on the Kv4.2 subunit might explain the involvement of MAPKs in the acute responses to excitatory agonists in the present study: p44/42 MAPK (ERK1/2) can phosphorylate and inactivate the Kv4.2 subunit (38). In cell culture, agonist-induced phosphorylation of p44/42 MAPK (9) and p-p44/42 MAPK-induced phosphorylation of Kv4.2 (1) occur within minutes. This time course is consistent with the SDF-1-induced pressor responses and with the ability of the p44/42 MAPK inhibitor to block the acute responses to SDF-1 in this study and to ANG II and TNF-α in our previous studies (55, 57, 59, 64). In the PVN, in which the normal GABergic regulation of neuronal excitation is reduced in heart failure (67), these MAPK-mediated effects on Kv function may well exacerbate the state of neurohumoral excitation.

**Limitations of the study.** Other chemokines and their receptors - most notably MCP-1/CCL2 (4, 5) and fractalkine/CX3CL1 (36, 58) - are present in cardiovascular regions of the brain, including the PVN. The present study focused only on SDF-1, and only on the PVN, to better understand the potential role of chemokines in sympathetic regulation. This approach was based on our previous study (59) demonstrating that SDF-1 is upregulated in the PVN in heart failure and that bilateral microinjection of SDF-1 into the PVN elicits exaggerated increases in MAP, HR, and RSNA responses in rats with heart failure. The results support the hypothesis that the chemokine SDF-1 has a potentially important role in ANG II- and TNF-α-driven sympathetic excitation in heart failure. Further studies may well establish a role for other chemokines neurohumoral regulation.

The present study did not establish the identity of the receptor mediating the responses to SDF-1. In our previous study, the selective antagonist for CXCR4, AMD 3100, was ineffective in blocking the hemodynamic and sympathetic responses to ICV SDF-1, and a selective antagonist for CXCR7 is not currently available. Thus, to answer the question of whether SDF-1 contributes to ANG II and TNF-α-driven sympathetic excitation in heart failure. Further studies may well establish a role for other chemokines neurohumoral regulation.

We did not quantify the extent to which ICV SDF-1 shRNA reduced SDF-1 protein in the PVN. However, in a previous study (59), the same dose of SDF-1 shRNA normalized the increased SDF-1 expression in rats with heart failure while reducing their plasma arginine vasopressin, adrenocorticotrophic hormone, and norepinephrine levels. In the present study, RT-PCR (Fig. 6) studies revealed that the same ICV dose of SDF-1 shRNA reduced TNF-α and ANG II-induced SDF-1 mRNA in PVN. The reduction in ANG II- and TNF-α-induced immunofluorescent reactivity for p-p44/42 MAPK in the PVN (Fig. 5) is further evidence of the effectiveness of the SDF-1 shRNA. Immunofluorescent studies revealed that SDF-1 shRNA reduced TNF-α-induced SDF-1 expression in PVN, subfornical organ, and supraoptic nucleus (Fig. 7). Although it is conceivable that SDF-1 knockdown in other cardiovascular/autonomic regions of the brain may have contributed to the observed reduction in p-p44/42 MAPK in the PVN, the microinjection data strongly suggest that SDF-1 in the PVN stimulates sympathetic activity via activation of p44/42 MAPK.

Finally, one may ask whether ANG II has a direct effect to increase SDF-1 expression in the PVN or whether its effect is indirect via the induction of pro-inflammatory cytokines (26). The present study did not examine this possibility.

**Perspectives.** Inflammatory chemokines play an important role in neuroinflammatory states (35). Studies over the past decade have established central inflammation as a prominent feature of heart failure and hypertension (22, 39, 66). We recently demonstrated upregulation of the chemokine SDF-1 in cardiovascular regions of the brain in heart failure (59). The present study suggests a viable explanation for an upregulation of SDF-1 in that setting, in which brain renin-angiotensin system activity and pro-inflammatory cytokines are increased (15, 22, 27, 47, 62). It also identifies MAPK signaling as a critical process in SDF-1-mediated sympathetic excitation and raises the possibility that SDF-1 may account, at least in part, for the ANG II- and the proinflammatory cytokine-induced MAPK signaling that leads to sympathetic excitation in heart failure and hypertension.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

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