c-Fos expression in rat brain stem and spinal cord in response to activation of cardiac ischemia-sensitive afferent neurons and electrostimulatory modulation

Fang Hua, Theresa Harrison, Chao Qin, Angela Reifsteck, Brian Ricketts, Charles Carnel, and Carole A. Williams.

Departments of Physiology and Anatomy and Cell Biology, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614-1708; and Department of Physiology, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

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Hua, Fang, Theresa Harrison, Chao Qin, Angela Reifsteck, Brian Ricketts, Charles Carnel, and Carole A. Williams. c-Fos expression in rat brain stem and spinal cord in response to activation of cardiac ischemia-sensitive afferent neurons and electrostimulatory modulation. Am J Physiol Heart Circ Physiol 287: H2728–H2738, 2004. First published July 29, 2004; doi:10.1152/ajpheart.00180.2004.—The purpose of this study was to identify central neuronal sites activated by stimulation of cardiac ischemia-sensitive afferent neurons and determine whether electrical stimulation of left vagal afferent fibers modified the pattern of neuronal activation. Fos-like immunoreactivity (Fos-LI) was used as an index of neuronal activation in selected levels of cervical and thoracic spinal cord and brain stem. Adult Sprague-Dawley rats were anesthetized with urethane and underwent intrapericardial infusion of an “inflammatory exudate solution” (IES) containing allogenic substances that are released during ischemia (10 mM adenosine, bradykinin, prostaglandin E2, and 5-hydroxytryptamine) or occlusion of the left anterior descending coronary artery (CoAO) to activate cardiac ischemia-sensitive (nociceptive) afferent fibers. IES and CoAO increased Fos-LI above resting levels in dorsal horns in laminae I–V at C2 and T4 and in the caudal nucleus tractus solitarius. Dorsal rhizotomy virtually eliminated Fos-LI in the spinal cord as well as the brain stem. Neuro-modulation of the ischemic signal by electrical stimulation of the central end of the left thoracic vagus excited neurons at the cervical and brain stem level but inhibited neurons at the thoracic spinal cord during IES or CoAO. These results suggest that stimulation of the left thoracic vagus excites descending inhibitory pathways. Inhibition at the thoracic spinal level that suppresses the ischemic (nociceptive) input signal may occur by a short-loop descending pathway via signals from cervical proprioceptive circuits and/or a longer-loop descending pathway via signals from the dorsal motor nucleus of the vagus, and sympathetic thoracic intermediolateral cell columns (17) as part of the circuitry processing this noxious cardiac visceral information.

The aims of the present study were twofold. The two major experimental models of cardiac ischemia described above have not been compared as to the detailed patterns of central neuronal activation they individually produce. Therefore, the first aim was to identify the sites in the cervical and thoracic spinal cord and caudal brain stem, especially in the NTS, activated by cardiac ischemia-sensitive (nociceptive) afferent neurons (CISAN) by the two procedures using changes in c-Fos immunoreactivity. The second aim of this study was to determine whether thoracic vagal afferent stimulation altered the patterns of neuronal activation in the spinal cord and brain stem, because it is well documented that electrical stimulation of the left thoracic vagus results in decreased electrical activity of STT neurons in the thoracic spinal cord that were excited by noxious stimuli (1, 6, 13, 33, 39). This is important to document, because electrical stimulation of left vagal afferent fibers has been used therapeutically to reduce cardiac pain (13, 42).

MATERIALS AND METHODS

Surgical Preparation of Animals

Sprague-Dawley rats of either gender (n = 41, 288 ± 53 g body wt; Harlan, Indianapolis, IN) were initially anesthetized with urethane...
Surgical-level anesthesia was maintained throughout the experiment via supplemental injections (30 mg·kg⁻¹·h⁻¹) through the right jugular vein. All experiments and procedures were reviewed and approved by the Institutional Committee on Animal Care and conformed to the Animal Welfare Act according to the Public Health Policy on Humane Care and Use of Laboratory Animals. Arterial pressure (BP) was measured directly via a catheter inserted into the left femoral artery and connected to a pressure transducer (Grass Ohmeda P23 XL-1). A tracheotomy was performed, and all rats were ventilated using a small-animal ventilator (Harvard Instruments) (frequency 70–80/min, tidal volume = 2–3 ml). Heart rate (HR) was determined from the pressure pulse signal or the ECG recording. Body temperature was maintained by placing the animal on a heating pad.

Cardiovascular data are presented as HR and mean blood pressure (MBP), calculated as diastolic BP + one-third pulse pressure. Values are means ± SE. The changes measured in response to a particular intervention or after the intervention were averaged for each animal and entered in the group mean. Significance was determined (SigmaStat) from Student’s t-test for paired data within a specific experimental group, using one-way ANOVA between groups, followed by Tukey’s post hoc tests for pairwise comparisons. The nonparametric data were treated with Kruskal-Wallis one-way ANOVA on ranks followed by Dunn’s test for post hoc comparisons. P ≤ 0.05 is taken as the minimum level of significance.

Rats were divided into groups to determine the neuronal activation at the spinal and selected brain stem levels in response to 1) the effects of exciting cardiac ischemic afferent fibers, i.e., by inflammatory exudate solution (IES) or occlusion of the left anterior descending coronary artery (CoAO); 2) the effects of eliminating afferent input to the spinal cord, i.e., dorsal rhizotomy; 3) the effects of nonischemic cardiovascular afferent input, i.e., baroreceptor-related input [produced by intravenous phenylephrine (PE) or carotid artery occlusion (CAO)]; and 4) the effects of electroneurostimulatory modulation of

Fig. 1. Fos immunoreactivity in rat spinal cord. Photomicrographs show Fos immunoreactivity at T₄ spinal level. A: rest; B: left thoracic/cervical vagal stimulation (LVS); C: intrapericardial infusion of inflammatory exudate solution containing algogenic substance (IES); D: occlusion of left anterior descending coronary artery (CoAO); E: IES after dorsal rhizotomy (IES + DR); F: CoAO after dorsal rhizotomy (CoAO + DR); G: IES with simultaneous electrical stimulation of the central end of the sectioned left thoracic/cervical vagus (IES + LVS); H: CoAO + LVS. Scale bar in G (100 μm) applies to A–H.
the input signal (produced by left vagal stimulation). Each of these main groups had appropriate controls (see below). Because we did not observe any differences in the cardiovascular responses or Fos-like immunoreactivity (Fos-LI) to these procedures between male and female rats, data from male and female rats were pooled.

**Intrapericardial infusion of IES.** After the basic surgical preparation for blood pressure measurement and ventilation, the rats (n = 3) receiving intrapericardial infusions of IES underwent a short midster nal incision to expose the thymus. The thymus was separated for a short length at midline to expose a small opening into the pericardial sac, through which a small length of silicone tubing (0.25 in. ID, 0.47 in. OD) was inserted into the opening, to abut the surface of the heart toward the apex. The catheter was filled with 0.2–0.5 ml of warmed normal saline and flushed regularly throughout the rest period by slow infusion of 0.2 ml of saline and then withdrawal of 0.2 ml of fluid. During the infusion of algogenic substances, 0.2 ml of IES was infused through the catheter over 20–30 s and remained in contact with the surface of the heart for 2 min. This solution was gently withdrawn over 20–30 s, and 0.2 ml of normal saline was then infused into the pericardial sac for 2 min. This infusion-withdrawal of IES-saline was repeated for a total of three infusions every 12 min. After a 10-min rest period, the entire protocol was repeated two more times over the course of 1 h. The IES contained 10 mM adenosine, bradykinin, prostaglandin E2, and 5-hydroxytryptamine. As a control over the course of 1 h. The IES group, in a separate group of rats (n = 3), normal saline was repeatedly infused and withdrawn over the course of 1 h as described for the IES. At the completion of the infusions and the subsequent 90-min rest period, a small amount (0.2 ml) of Pontamine blue dye was infused into the pericardial sac through the catheter to verify that the tip of the catheter was within the pericardial sac and that there was no leakage of the IES.

**CoAO.** For the rats that would undergo coronary artery occlusions (n = 4), after the initial basic preparation for ventilation and blood pressure recording, a left thoracotomy was performed at the fourth rib. A segment of saline-soaked 5-0 suture was looped around the left anterior descending coronary artery near its branch point from the left coronary artery, and the ends of the suture were passed through a 1-in. segment of double-barreled polyethylene tubing. The ends of the tubing were rounded, so that no rough-surface tubing would damage the coronary artery. The tubing was gently placed next to the heart and secured by three knots tied on the suture at the external end of the section of tubing, with the last knot up against the end of the tubing. After completion of this procedure, the polyethylene tubing-and-suture assembly was exteriorized, and the thorax was closed. Coronary artery occlusion was accomplished by advancing the last knot up against the external end of the tubing 2 mm away from the tubing by inserting the tip of surgical tweezers between the last knot and the tubing and gently opening the tips 2 mm apart. This permitted reproducible occlusion of the left anterior descending coronary artery without laceration of the artery. The CoAO was applied for 90 s with a 60-s rest interval, with four occlusions applied over a 10-min period. This procedure was repeated two more times over the course of 1 h after interspersed 10-min rest periods.

**Dorsal rhizotomy.** In another group of animals, dorsal rhizotomy was performed to determine whether elimination of afferent input into dorsal horn laminae affected neuronal activation. During this procedure, thoracic spinal segments T1–T6 were exposed and their lateral processes were removed. Tubocurarine (67 μg/kg body wt iv) was injected before the dura and pia mater were removed from these segments. The dorsal roots of spinal segments T2–T6 were identified, gently separated, and then sectioned bilaterally, close to the lateralmost area of each segment. After completion of this additional surgical preparation and a 60-min rest period, some animals underwent the CoAO protocol (n = 5) and others underwent the IES protocol (n = 3).

**Noncardiac ischemic controls.** A rest group of rats (n = 3) underwent the same basic surgical preparation as all other rats but were not subjected to further procedures. Fos-LI measured in these animals served as the “rest” control. Two additional separate groups of rats were used as controls in these experiments to determine whether any baroreflex-induced changes in Fos-LI appeared in sites similar to the IES- or CoAO-induced Fos. One group (n = 3) underwent temporary bilateral CAO, where the common carotid

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**Fig. 2.** Quantification of c-Fos-positive sites in T4 spinal segment. Fos-positive cells were counted under bright-field microscopy within laminae I–V for left and right sides of T4 spinal segments and then expressed as a function of cross-sectional area of laminae I–V. A: controls, including sham surgery (rest) and noncardiac ischemic afferent input, involving baroreceptor unloading, bilateral carotid artery occlusion (CAO), and baroreceptor activation by intravenous phenylephrine (PE) injection. B: response to cardiac ischemia-sensitive afferent neuron (CISAN) activation. C: electrostimulatory modulation involving electrical stimulation of left thoracic/cervical vagus. Rest in B and C is the same as in A and is presented for comparison. Height of each bar represents average number of Fos-positive cells; error bars represent SE. *P < 0.05 vs. right. & P < 0.05 vs. rest. & P < 0.05 vs. corresponding IES or CoAO alone.
arteries below the bifurcation of the internal and external carotid arteries were clamped for 20 s every 2 min for a total of 8–10 repeats over the course of 30 min. This procedure temporarily unloaded the carotid baroreceptors, causing a reduction in carotid afferent input, resulting in a reflex increase in HR. In another group of rats (n = 3), a slow (10–15 s) intravenous injection of PE was administered through the jugular vein (0.02 ml of 1 mM PE) every 10 min over the course of 60 min. This α-adrenoceptor agonist induced peripheral vasoconstriction, raising BP and, thereby, activating arterial mechanosensory afferent neurons to reflexively decrease HR.

**Left vagal stimulation.** To determine whether left vagal stimulation (LVS) modified spinal dorsal horn or brain stem neuronal activation in response to IES or CoAO, three different groups of rats underwent electrical stimulation of the left thoracic vagus alone (LVS, n = 5), intrapericardial infusion of IES during LVS (IES + LVS, n = 3), or coronary artery occlusion during LVS (CoAO + LVS, n = 3). In this set of experiments, after the basic surgical preparation for blood pressure measurement and ventilation, the left vagosympathetic trunk was identified in the midline incision made for tracheal intubation and separated from the carotid artery and vein. The sympathetic nerve trunk and any sympathetic nerve branches around the vagus were freed from the vagus. A 5-0 suture was tied around the left vagus at the thoracic level (T1–T2). A bipolar platinum electrode (27 gauge) was positioned rostrally under this area of the vagus and secured with a small drop of dental acrylic. The right vagus was sectioned at the cervical-thoracic junction, and then the left vagus was sectioned caudally to the suture. A few drops of mineral oil were placed around the electrode-left vagal preparation, and the skin was sutured closed. A group of three rats were prepared to this point and used as the control [i.e., bilateral vagotomy (BV)] for the LVS groups of rats. These control animals did not undergo any electrical stimulation. LVS was accomplished by application of 10-Hz pulses of 2.5-ms duration at 10 μA (5.0 V) via a Grass stimulator (model S48) connected to a constant-current stimulus isolation unit (model SIU 7, Grass). Stimulation was applied for 90 s every other 60 s for 10 min. The animals were allowed 10 min of rest; then the procedure was repeated two more times over the course of 1 h. For the experiments involving LVS + CoAO or LVS + IES, the LVS was applied first; then after 5–10 s, the ischemic procedure (IES or CoAO) was applied as described above.

![Figure 3](http://ajpheart.physiology.org/)

**Fig. 3.** Noncardiac ischemic afferent induction of Fos immunoreactivity. Photomicrographs show c-Fos in response to CAO or PE in T4 spinal segment (A and B), C2 spinal segment (C and D), and dorsal caudal brain stem in nucleus tractus solitarius (NTS) region (E and F).
Immunohistochemistry

After completion of the experimental period, animals were given a large dose of urethane (300 mg/0.5 ml) and then perfused transcardially with 500 ml of normal saline using a peristaltic pump (Preston Manostat) followed by 500 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Blocks of medulla and spinal cord were removed, postfixed in 4% paraformaldehyde at room temperature for 1.5–2.0 h, and then stored overnight at 4°C in a solution of 30% sucrose-PBS. The T4 segment was identified by deposition of a small dot of Pontamine blue dye on the dorsal surface of the spinal cord before it was removed. Consecutive transverse 40-μm sections were cut with a cryostat (IEC) at −20°C and transferred immediately to five alternate wells of polypropylene plates containing PBS.

Sections were washed twice in PBS for 15 min each and then incubated in 3% H₂O₂ for 10 min to quench endogenous peroxidase activity. The sections were washed again three times for 15 min each in PBS. Sections were then incubated in a blocking solution containing 1% normal goat serum, 0.4% Triton X-100, and 0.5% BSA in PBS for 1 h at room temperature. The sections were then incubated in c-Fos antibody from rabbit (1:1,000 in PBS; AB-5, Oncogene) for 48 h at 4°C with constant gentle shaking. There was no cross-reactivity with Jun protein (Oncogene). At the completion of this incubation, the sections were washed in cold PBS three times for 15 min each at room temperature and incubated with biotinylated anti-rabbit IgG (1:200 in 1% normal goat serum, 0.4% Triton X-100, and 0.5% BSA in PBS) for 2.5 h at room temperature. The sections were then incubated in c-Fos activity. The sections were washed again three times for 15 min each at room temperature and incubated with biotinylated anti-rabbit IgG (1:200 in PBS with 0.4% Triton X-100 and 0.5% BSA) for 2.5 h at room temperature. The sections were then washed twice in PBS for 15 min each and then incubated in 3% H₂O₂ for 10 min to quench endogenous peroxidase activity. The sections were washed again three times for 15 min each in PBS. Sections were then incubated in a blocking solution containing 1% normal goat serum, 0.4% Triton X-100, and 0.5% BSA in PBS for 1 h at room temperature. The sections were then incubated in c-Fos antibody from rabbit (1:1,000 in PBS; AB-5, Oncogene) for 48 h at 4°C with constant gentle shaking. There was no cross-reactivity with Jun protein (Oncogene). At the completion of this incubation, the sections were washed in cold PBS three times for 15 min each at room temperature and incubated with biotinylated anti-rabbit IgG (1:200 in 1% normal goat serum, 0.4% Triton X-100, and 0.5% BSA in PBS) for 2.5 h at room temperature. The sections were then incubated in c-Fos buffer (pH 7.6) three times for 15 min each, and Fos-LI was visualized by incubation with 10 ng of 3′,3′-diaminobenzidine and 3% H₂O₂ in Tris buffer. The reaction was stopped after 3 min by transfer of the sections to Tris buffer (pH 7.6). Sections were subsequently transferred to a 1% alcoholic gelatin solution, mounted on slides, and air-dried overnight, and coverslips were applied.

Localization of Fos-LI was evaluated by bright-field microscopy using an Olympus BH2 microscope. Sites in the central nervous system (CNS) were identified using the atlas of Paxinos and Watson (26). Quantification of the Fos-positive cells was done by counting the number of dark brown nuclei in a 0.01-mm² area on either side of the midline (determined by microscope calibrated reticule) within the NTS in the brain stem for each of five alternate sections at the specific level (bregma −13.8 mm) for a particular rat. Fos-positive cells were counted in the dorsal horn in laminae I–V in five alternate spinal cord sections at T₄ or T₅ for each animal. Data were then grouped and averaged for each experimental category. The number of immunoreactive cells in the dorsal horn (laminae I–V) was expressed per square millimeter. The surface area was determined using the basic area morphometry function of MCID Imaging Research software. The average surface area of laminae I–V was 0.41 ± 0.02 and 0.18 ± 0.01 mm² in C₂ and T₄, respectively. Summary data for Fos-positive cells were generated from the raw data and compared using one-way ANOVA. Subsequent treatment of between-group comparisons and significance levels were as described above for the cardiovascular data.

RESULTS

Fos Expression in Thoracic Spinal Cord

There was little Fos expression in animals that remained at rest after basic surgery (Fig. 1A). Fos-positive neurons were randomly dispersed throughout the dorsal horn and equally distributed in the left and right sides of the thoracic spinal cord at T₄ (Fig. 2A). Noncardiac ischemic afferent input in the form of CAO or PE injection did not alter the number of Fos-positive neurons in the thoracic spinal cord dorsal horn, laminae I–V (Fig. 3, A and B). Baroreceptor unloading during CAO elicited neuronal activation in lamina X around the central canal (Fig. 3A).

In contrast, stimulation of cardiac ischemia-sensitive afferent fibers by intrapericardial infusion of IES (Fig. 1C) or CoAO (Fig. 1D) increased Fos-LI in the dorsal horns of thoracic spinal segments by nearly four times above the rest levels [F (df 2) = 18.77, P < 0.001]. The IES procedure induced significantly higher levels of Fos than pericardial saline infusion (Fig. 2B). It is apparent from Fig. 4 that more Fos-positive cells were found in the lateral portion of the dorsal horns in the T₄ segment during IES than during CoAO and more Fos-positive cells were found medially during CoAO than during IES. However, both procedures activated a similar number of neurons in laminae I–V in the left dorsal horn at T₄ (Fig. 2B), and more neurons were activated in the left than in the right dorsal horn (Fig. 2B). IES or CoAO after dorsal rhizotomy of spinal segments T₂–T₅ significantly reduced Fos-LI in the thoracic dorsal horn compared with either procedure with intact spinal roots (Fig. 1, E and F).

Electrostimulatory modulation of the cardiac ischemic input signal at the thoracic level had an inhibitory effect on the number of Fos-positive neurons. Neither bilateral vagotomy (the sham surgical control procedure for LVS) nor stimulation of the central end of the cut left thoracic vagus (LVS) alone increased Fos-LI in the T₄ spinal segment significantly above rest levels (Fig. 2C; P = 0.10). There was no difference in Fos expression between the right and left dorsal horns in response to bilateral vagotomy. IES + LVS significantly reduced the number of Fos-positive cells in the T₄ segment, laminae I–V, compared with IES alone (Figs. 1G and 2C). However, Fos expression was higher around the central canal, in lamina X, during the combined procedures. LVS + CoAO significantly decreased Fos-LI in dorsal horn laminae I–V compared with...
CoAO alone (Figs. 1H and 2C), although, again, the number of Fos-positive cells around the central canal, in lamina X, was increased.

**Fos Expression in Cervical Spinal Cord**

The number of Fos-positive neurons in the C2 spinal segment during rest (Figs. 5A and 6A) was similar to that in the thoracic spinal segment per unit area (Figs. 1A and 2A). Neither unloading the arterial baroreceptors by bilateral CAO (Fig. 3C) nor activating the baroreceptors by intravenous injection of PE (Fig. 3D) had a significant effect on basal Fos expression in the C2 segment dorsal laminae I–V [Fig. 6A; $F(2,5) = 2.86, P = 0.10$]. There was no difference in the number of Fos-positive cells in the left or right dorsal horn at rest, with CAO, or with PE injection (Fig. 6A).

As in the thoracic spinal cord, activation of CISAN by either method (Fig. 5, C and D) increased Fos expression above rest levels in the cervical dorsal horns. However, IES caused significantly more Fos expression at the cervical level than did CoAO (Fig. 6B). This neuronal activation was caused by activation of CISAN, because dorsal rhizotomy significantly reduced Fos expression in response to IES and CoAO (Figs. 5, E and F, and 6B).

In contrast to the thoracic spinal cord, stimulation of the central end of the left thoracic vagus by itself (LVS) significantly increased Fos-LI above resting levels (Figs. 5B and 6C) equally in the left and right dorsal horns. The sham control animals for the LVS surgery (bilateral vagotomy) were not different from rest controls (Fig. 6C). LVS during IES did not change the high level of neuronal activation at C2 resulting from IES.
Fos Expression in the Brain Stem

Fos expression was visualized in the dorsal medulla, at bregma −13.6 mm to −13.8 mm. Figure 7A illustrates background Fos expression in the dorsal central area, where the area postrema, NTS, and dorsal motor nucleus are found, in sections from animals remaining at rest. The number of active cells within the NTS was similar in the left and right sides of the brain stem (Fig. 8A). The nonischemic procedures (CAO and PE) had opposite effects on the number of Fos-positive cells in the NTS. Baroreceptor unloading by CAO reduced the Fos-positive cells in the NTS compared with rest levels (Figs. 3E and 8A), whereas baroreceptor activation by PE injection increased Fos expression in the NTS compared with resting conditions (Figs. 3F and 8A). These effects were similar on the left and right sides of the brain stem (Fig. 8A).

Activation of CISAN by the IES or CoAO method significantly increased the number of Fos-positive neurons at this level of the brain stem relative to rest and saline infusion (Figs. 7, C and D, and 8B). Although not quantified, IES infusion appeared to greatly increase the number of Fos-positive neurons in the area postrema as well (Fig. 7C). Fos expression in the brain stem during IES and CoAO was dramatically reduced after bilateral dorsal rhizotomy (Fig. 7, E and F, and 8B).

Electrostimulatory modulation of the CISAN input signal had opposite effects on neuronal activation within the NTS, depending on the method of activation of CISAN. Bilateral vagotomy controls did not show significant changes in Fos expression in the NTS (Fig. 8C), but LVS alone resulted in increased levels of Fos compared with rest (Figs. 7B and 8C). Although IES + LVS produced more Fos-positive cells than LVS alone, IES + LVS did not cause a greater number of Fos-positive cells than IES alone (Fig. 8C). Similarly, LVS + CoAO did not significantly affect the number of Fos-positive cells in the NTS compared with CoAO alone (Fig. 8, B and C).

Cardiovascular Responses to CISAN Activation

The cardiovascular changes resulting from activation of CISAN are presented in Table 1. Neither MBP nor HR varied significantly over the duration of an entire experiment in the rest control animals. Repeated intrapericardial infusion or withdrawal of saline did not alter MBP or HR in the other control group. In general, the procedures used to excite CISAN or the controls for these procedures caused only modest alterations in MBP and HR from their resting levels. For example, there was a consistent decrease in MBP from its preinfusion rest level during IES, but this decrease was not significant (P = 0.27), whereas withdrawal of IES returned MBP to resting levels.

DISCUSSION

Activation of CISAN by intrapericardial infusion of a solution containing algogenic substances (IES) or by occlusion of the left anterior descending coronary artery (CoAO) causes an increase in Fos expression in the spinal cord (at cervical and thoracic levels) and caudal brain stem, particularly in subnuclei of the NTS. The cells demonstrating increased expression of Fos are found at the neuroaxis levels previously identified by electrophysiological studies as being activated in response to CISAN stimulation (8, 13, 17, 21, 23, 30, 37). To our knowledge, this is the first report to localize Fos-positive cells at different sites in the CNS depending on the method used to activate CISAN. This is most apparent at the thoracic level, where IES tended to excite more cells in the lateral portion of the cord.
the dorsal horns, whereas CoAO excited more cells in the medial portion of the thoracic spinal segment. Our results also show that left thoracic vagal afferent stimulation increases the number of Fos-positive neurons at the cervical spinal level but decreases the number of active neurons at the thoracic spinal level. Even though we cannot measure alleviation of the discomfort caused by CISAN activation in an anesthetized animal model, we know from previous studies (11) that activation of these cardiac afferent fibers is associated with behavior consistent with pain. CISAN excitation is the initiating event for transmitting the pain signal associated with cardiac ischemia (11, 13), and electroneuromodulation diminishes this signal. Thus our data suggest that the effects of LVS in blocking pain may be predominantly localized to thoracic spinal circuits.

The IES procedure exposes the entire epicardium to the increased concentration of algogenic substances simultaneously, presumably causing diffuse activation of many, if not all, of the most superficial cardiac ischemia-sensitive fiber endings in the epicardium. In contrast to the IES procedure, the CoAO procedure causes a focal and intense ischemic area within the myocardium. It is not surprising that increases in Fos-positive cells were located in the dorsal horns after either of these procedures, inasmuch as other forms of visceral inflammation (e.g., mustard oil irritation of the colon) cause increased expression of Fos, mainly in the superficial laminae.
of L₆ and S₁ spinal segments and, to a lesser extent, in deeper spinal laminae (24, 25, 34). That the observed effects are specific to CISAN activation is supported by the lack of increases in Fos expression in the saline controls, indicating that activation of lower-threshold chemo- or mechanosensitive fibers was not the cause of the observed changes. Second, the nonischemic afferent signaling, involving arterial baroreceptor procedures, did not cause any significant Fos expression at the cervical or thoracic level. Furthermore, the upper thoracic dorsal rhizotomy virtually eliminated Fos expression in the cervical and thoracic dorsal horns as well as in the NTS in response to either procedure, in agreement with previous evidence (8, 13, 22, 23, 25) that the majority of the sensory neurons responsive to cardiac ischemia are located in dorsal root ganglia between T₁ and T₆. Although there is evidence that nodose ganglion cardiac afferent neurons also transmit ischemic signals, only ~10% of these neurons are sensitive to the mechanical and chemical stimuli associated with cardiac ischemia (2). The very low levels of Fos in the cervical and thoracic spinal segments, even with the use of urethane, an anesthetic noted to result in high background levels of Fos in the brain stem and other higher centers (36), indicate that the differences in Fos expression were clearly a function of the experimental interventions and not the anesthetic.

Thoracic respiratory interneurons receive noxious somatic and visceral input (27, 30), including cardiac afferent input (28), especially those in the intermediate zone and deeper lamina. Therefore, it is possible that some proportion [i.e., 27–40% (28)] of activated neurons identified by Fos-LI might be respiratory-related neurons responding to noxious input from CISAN in our experiments. This may account for the differences in the distribution of Fos-positive cells between the intermediomedial segments of the thoracic and cervical spinal cord.

It was not surprising that CISAN activation by IES or CoAO caused an increased neuronal activation in the caudal brain stem. The events in the NTS were quantitated, because the NTS receives direct activation from cardiac “nociceptive” afferent neurons via vagal afferent fibers (13, 23). However, the drastic reduction in Fos expression in NTS neurons after dorsal rhizotomy suggests that these direct afferents are not essential for the observed responses to IES and CoAO. Furthermore, noncardiac ischemic afferent vagal input to the NTS primarily activated neurons in the dorsomedial NTS, as others have shown (10, 34), whereas IES and CoAO resulted in Fos increases in the dorsomedial, ventromedial, dorsolateral, and commissural NTS. The observed increases in Fos in the NTS may reflect, in part, excitation of neurons by reciprocal descending projections from the medial thalamus, anterior cingulated gyrus (13), and ventrolateral and lateral periaqueductal gray (15). The loop from activated CISAN through the periaqueductal gray to excite neurons in the NTS has been previously described (13, 23). The present experiments cannot distinguish among the various input possibilities.

The differences we observed in localization patterns of Fos-positive cells after the two procedures were not likely due to magnitude differences in the initiating stimulus. If the consequences of CISAN activation were a function of stimulus magnitude (i.e., total number of CISAN endings excited), then we would have expected consistent differences in the number of Fos-positive cells after IES compared with CoAO at all three neural sites examined. We did not see a consistent difference. The number of Fos-positive neurons in the T₄ segment and in the NTS is roughly the same for IES and CoAO but is greater at the C₂ level for IES than for CoAO. Thus it is more likely that these differences in neuronal activation are due to the nature of the initiating stimulus, the localization of the activated afferent fiber in the heart, or the selective type of afferent

Fig. 8. Quantification of c-Fos-positive sites in nucleus tractus solitarius (NTS). Number of Fos-positive neuronal sites in NTS was determined under bright-field microscopy within a 100 x 100 μm square grid area. Height of each bar represents average; error bars represent SE. *P < 0.05 vs. rest. †P < 0.05 vs. right. ‡P < 0.05 vs. IES or CoAO alone; §P < 0.05 vs. CoAO alone. ¶P < 0.05 vs. IES + LVS or CoAO + LVS. A: brainstem NTS; B: CISAN activation; C: electrostimulatory modulation.
fiber stimulated in response to the initiating signal. This conclusion is supported by previous findings from this laboratory that CoAO induces the release of substance P from laminae I–VII in the thoracic spinal cord, but IES does not (16).

Effects of LVS on Fos Expression in Response to CISAN Activation

Vagal afferent stimulation alone increased Fos expression above rest in the NTS, most likely because of activation of direct vagal afferent projections to this nucleus. LVS + IES produced a total of Fos-positive cells that was greater than that produced by LVS or IES alone. This may be the result of simply activating more afferent fibers that have input into the NTS than could be activated by either procedure alone. In contrast, the effects of CoAO were not further enhanced by simultaneous LVS, perhaps because the number of neurons activated by the strong CoAO signal was already high.

Only in the thoracic spinal cord did application of LVS during IES or CoAO reduce the number of Fos-positive cells compared with the two procedures alone. Activity of the vagal afferent neurons was necessary for this effect, as inasmuch as bilateral vagotomy did not produce a similar change. These data support previous electrophysiological reports of decreased excitation of STT cells in the thoracic spinal cord (1, 30–33, 39), in contrast to increased excitation in upper cervical spinal cord (1, 30–33, 39), in contrast to increased excitation in upper cervical spinal cord (1, 30–33, 39). These data support previous electrophysiological reports of decreased excitation of STT cells in the thoracic spinal cord (1, 30–33, 39), in contrast to increased excitation in upper cervical spinal cord (1, 30–33, 39), in contrast to increased excitation in upper cervical spinal cord (1, 30–33, 39).


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Table 1. Cardiovascular changes during CISAN activation

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<th>MBP, mmHg</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Procedure</td>
</tr>
<tr>
<td>Rest</td>
<td>3</td>
<td>122±10</td>
</tr>
<tr>
<td>Saline</td>
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<td>86±15</td>
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<tr>
<td>IES</td>
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<td>98±9</td>
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<tr>
<td>CoAO</td>
<td>4</td>
<td>105±10</td>
</tr>
<tr>
<td>IES + DR</td>
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<tr>
<td>CoAO + DR</td>
<td>5</td>
<td>78±5</td>
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<tr>
<td>LVS</td>
<td>5</td>
<td>107±5</td>
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<td>121±17</td>
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<tr>
<td>CoAO + LVS</td>
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<td>122±4</td>
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</tbody>
</table>

Values are means ± SE. CISAN, carotid ischemia-sensitive afferent neuron; MBP, mean blood pressure; HR, heart rate; IES, inflammatory exudate solution; CoAO, occlusion of left anterior descending coronary artery; LVS, left vagal stimulation; DR, dorsal rhizotomy. *P < 0.01 vs. corresponding Rest.
Fos EXPRESSION IN CNS DURING CARDIAC ISCHEMIA