Modulation of cardiac ischemia-sensitive afferent neuron signaling by preemptive C2 spinal cord stimulation: effect on substance P release from rat spinal cord

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Ding XH, Ardell JL, Hua F, McAuley RJ, Sutherly K, Daniel JJ, Williams CA. Modulation of cardiac ischemia-sensitive afferent neuron signaling by preemptive C2 spinal cord stimulation: effect on substance P release from rat spinal cord. Am J Physiol Regul Integr Comp Physiol 294: R93–R101, 2008. First published November 7, 2007; doi:10.1152/ajpregu.00544.2007.—The upper cervical spinal region functions as an intraspinal controller of thoracic spinal reflexes and contributes to neuronal regulation of the ischemic myocardium. Our objective was to determine whether stimulation of the C2 cervical spinal cord (SCS) of rats modified the input signal at the thoracic spinal cord when cardiac ischemia-sensitive (sympathetic) afferents were activated by transient occlusion of the left anterior descending coronary artery (CoAO). Changes in c-Fos expression were used as an index of neuronal activation within the spinal cord and brain stem. The pattern of substance P (SP) release, a putative nociceptive transmitter, was measured using antibody-coated microprobes. Two SCS protocols were used: reactive SCS, applied concurrently with intermittent CoAO and preemptive, sustained SCS starting 15 min before and continuing during the repeated intermittent CoAO. CoAO increased SP release from laminae I and II in the T4 spinal cord above resting levels. Intermittent SCS with CoAO resulted in greater levels of SP release from deeper laminae IV–VII in T4 than CoAO alone. In contrast, SP release from laminae I and II was inhibited when CoAO was applied during preemptive, sustained SCS. Preemptive SCS likewise reduced c-Fos expression in the T4 spinal cord (laminae I–V) and nucleus tractus solitarius but increased expression in the intermedio-lateral cell column of T4 compared with CoAO alone. These results suggest that preemptive SCS from the high cervical region modulates sensory afferent signaling from the ischemic myocardium.

antibody-coated microprobes; angina; nociceptive peptides; cardiac nervous system; c-Fos expression

ELECTROMODULATION THERAPY in the forms of either vagal afferent or spinal cord stimulation is reported to reduce episodes of intractable angina with no adverse cardiovascular side effects (6, 17, 27, 30, 38, 39, 43). Acute nociception is not blocked (29), and individuals are able to identify the onset of a myocardial infarction even though refractory angina is alleviated (2, 17–18). Thus, although either type of electrical stimulation exerts analgesic effects, the mechanisms involved with such approaches are not well understood and may not be identical.

Myocardial ischemia activates both vagal and sympathetic cardiac ischemia-sensitive afferent neurons (CISAN), and these sensory neurons transmit the nociceptive signal into the central nervous system (16, 25, 33). Given that differences exist regarding the location of the neurite cell bodies and various pathways excited by vagal and sympathetic CISAN (5, 10, 16, 17, 25, 28, 36, 37), it is not unexpected that the mechanisms responsible for cardiac sensory afferent signaling are not uniform and probably involve multiple peptide modulators interacting at a number of sites in the central nervous system. We previously reported that transient coronary artery occlusion (CoAO) activated sympathetic CISAN and excited neurons in laminae I–V in the cervical (C2) and thoracic (T4) spinal cord and brain stem (23). We also showed that transient coronary artery occlusion increased the release of immunoreactive substance P-like substances (irSP) above basal levels from laminae I–VII in spinal T4 segments (24) and that intermittent electrical stimulation of the left thoracic vagus applied coincident with occlusions, inhibited neurons at the T4 level (23) and attenuated the basal and CoAO-evoked release of SP from spinal T4 laminae I–VII (22), while causing a release of immunoreactive dynorphin-like substances (22).

The high cervical region plays an important regulatory role via propriospinal neurons in modulating the upper thoracic spinal processing of CISAN inputs (1, 10, 16, 17, 36). Specifically, direct electrical activation of the dorsal aspects of the C1–C2 spinal cord decreases the electrical activity of thoracic spinal neurons activated by input signals from cardiac ischemia-sensitive afferent neurons (16, 29, 31). We hypothesize that these C1–C2 regulatory effects reflect, in part, a neurally mediated reduction in neurotransmitter release from primary CISAN inputs. We further hypothesize that SP is a primary mediator of the nociceptive signal transmitted by cardiac ischemia-sensitive afferent neurons (22, 24) and that electromodulation exerts antinociceptive effects, in part, by inhibiting the release of SP from the thoracic spinal cord (22). This study was undertaken to determine 1) the effects of high cervical spinal cord stimulation (SCS) on afferent signaling of ischemia-sensitive neurons in the thoracic cord and brain stem as indexed by c-Fos expression; 2) the effects of high cervical SCS on ischemia-induced release of SP from sympathetic afferent projections into the thoracic spinal cord; and 3) to determine whether the timing of high cervical SCS, reactive vs. preemptive, had an impact on the efficacy of such neuromodulation at spinal and brain stem regions.
MATERIALS AND METHODS

Surgical preparation of animals. Male Sprague-Dawley rats (n = 54, 300.6 ± 6.2 g body wt; Harlan, Indianapolis, IN) were used in these studies. All procedures and experimental protocols were reviewed and approved by the East Tennessee State University Institutional Committee on Animal Care and Use and conformed to the Animal Welfare Act according to the Public Health Policy on Humane Care and Use of Laboratory Animals and adhere to the APS’s Guiding Principles in the Care and Use of Animals. Rats were anesthetized with urethane (1.5 g/kg body wt) injected intraperitoneally. Supplemental injections (30 mg/kg) of urethane were made every 90 min (or as needed) throughout the entire experiment, through the left femoral vein to maintain a surgical level of anesthesia. Rats were surgically prepared as described before (22–24). Cardiovascular data [pressure, heart rate, HR (beats/min, bpm)] were recorded and analyzed via PowerLab (version 4.24) and are presented as means ± SE. Data are reported for baseline, resting levels, for steady-state levels during an experimental intervention, and for recovery levels. Significance was determined from Student’s t-test for paired data within a specific experimental group and using one-way repeated-measures ANOVA between groups with follow-up comparisons using Tukey’s test. P < 0.05 was taken as the minimum level of significance.

Experimental groups. There were five different groups of rats undergoing one of the following protocols: transient, intermittent coronary artery occlusion (CoAO, n = 10); intermittent spinal cord stimulation [SCS protocol 1 (P1), n = 18]; sustained, continuous SCS (SCS P2, n = 9); simultaneously applied intermittent SCS with CoAO (SCS P1 + CoAO, n = 10), and preemptive, sustained SCS with intermittent CoAO [SCS protocol 2 (P2)] + CoAO, n = 7]. Only one experimental intervention was performed on a group of animals. Protocols are summarized in Fig. 1.

Laminectomy and microprobe placement. The spinal cord from C1–C3 and from T2–T4 was exposed on all of the rats by removing the appropriate vertebral processes. This was done to allow placement of a spring-loaded unipolar stimulating ball electrode on the dural surface at the C2 spinal level and for stereotaxic guided insertion of antibody-coated microprobes for SP at the T4 spinal level (22, 24). An intravenous injection of tubocurarine (67 μg/kg body wt) was given before removal of the dura and pia mater from the T4 spinal segment. Supplemental intravenous doses of urethane were continued every 90 min as described above, after the administration of tubocurarine and for the duration of the experiment.

Each of the in vivo probes was positioned at the rostrocaudal midpoint of the T4 spinal segment. Each probe was placed in the spinal cord with the aid of a digital micropositioner (Stoelting) and a stereotoxic surgical microscope with color video display. To ensure repeatability of their placement, each probe was visually positioned at the midline and surface of the spinal cord, moved 0.5 mm to the left of the midline, and inserted to a depth of 2.0 mm. Each probe remained in situ for 10 min. New probes were used for each 10-min pre-experimental or experimental procedure, and these were designated as “test” (Fig. 1, probes Pb1–Pb3), CoAO, SCS, or coronary occlusion with spinal cord stimulation (SCS + CoAO) (Fig. 1, Pb4–Pb6). At the completion of the 10-min in situ time, probes were withdrawn from the spinal cord and treated as described previously (22, 24, 42) and detailed below. Probe placement was verified by a deposit of Pontamine blue dye (e.g., see black dot in Fig. 5B), as described previously (22, 24, 42) using a stereotoxic atlas (35).

Measurement of immunoreactive SP using immobilized antibody microprobe technique. For each probe, the release of endogenous immunoreactive-SP from sites in the thoracic spinal cord was measured using the antibody-coated microprobe technique, as previously described (14, 22, 24, 42). In all experiments where probes were inserted into the thoracic spinal cord, a set of control probes (designated as in vitro probes) was identically and simultaneously prepared as the in vivo probes. The in vitro probes were used to determine the sensitivity of the binding of radiolabeled ligand (125I-Tyr8 SP, Phoenix Pharmaceuticals) (see Ref. 14 or 42) and to confirm the uniformity of binding of the silane and antibody along the probe shaft.

Image analysis of the microprobes. Autoradiographic images of the microprobes were analyzed for patterns of inhibition of binding of the radiolabeled ligand along the length of the probe. Such inhibition is indicative of where unlabeled (e.g., endogenously released) SP bound to the antibodies on the probe during the 10 min in vivo exposure time. Because the position of the probe tips was marked by a deposit of blue dye in the spinal cord, sites of release of the peptide could be determined from differences in the optical densities of the probe images on the autoradiographic film. The analysis was carried out based on initial methods described by Hendry et al. (21) and modified by this laboratory (22, 24, 42).

In the diagrams presented, the mean optical density of the probe image is converted to a grayscale in arbitrary units of 0–1026 (with 1026 being the darkest grayscale level). Each probe image was analyzed for 4 mm: the first 2 mm, starting at the tip, corresponded to the segment of the probe inserted into the spinal cord (designated as 2–0); while the next 2 mm corresponded to the part of the probe that remained outside the spinal cord (designated as 0 to −2 mm). The 2 mm outside the spinal cord served as an internal control area along each probe and for between-group controls via comparison to similar segments on the in vivo and in vitro probes. Typical pseudocolor images of microprobes were presented previously (22, 24, 42) together with a grayscale equivalent (43). The data presented in the image analysis figures are given as the mean grayscale levels ± SE for each specified group of probes. Software written in-house was based on the original statistical analysis described by Hendry et al. (21). The average grayscale levels are determined for the grouped probes.
starting at their tips, every 3 μm for the 4-mm image captured, calculates the means ± SE, and then plots this point to generate the image graph. Differences in the patterns of binding of radiolabeled SP along the probes during various experimental interventions were determined by Student’s t-test. This is done by position-specific independent comparisons between the same two points in the two groups of probes to determine whether the average grayscale levels at this point along the probes are different from each other. There is no multiple group comparison done between the two average lines. The calculated T value, where P = 0.05 (the minimum level of significance taken), is plotted along the lower portion of the image analysis graphs (just above the abscissa). The T-value for each pixel along the analyzed image was calculated and plotted in relation to the T-value of P = 0.05. Any points along the length of the probes that were different from each other appear above the T-value line. Because the resolution of detecting a difference in the binding of radiolabeled peptide is 100 μm (14), biological significance was defined only when the difference between two groups (i.e., the T-value) was maintained above the P = 0.05 line for a linear distance of at least 100 μm. The antibody-coated microprobe technique permits identification of sites within an area of the central nervous system, where a particular neuropetide is released. As such, this technique is used as a qualitative assessment of whether SP was released, what specific sites in the spinal cord released SP, and whether an experimental intervention (i.e., cardiac ischemia or spinal cord stimulation) altered the spatial pattern of SP release from basal control conditions within the T4 segment of the spinal cord. This technique is not used to determine differences in specific amounts of peptides released, and this should not be inferred from the graphic representation of the binding data presented (e.g., the height of the T-value above the significance line).

**Coronary artery occlusion.** A left thoracotomy was performed between the 4th and 5th ribs, while the animals were on ventilation. The pericardium was opened, and 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, as previously described (22, 24). A SP antibody-coated microprobe was positioned in the T4 spinal level for 10 min (rest, Pb1 probe) followed by a second rest probe (Pb2), and then third rest probe (Pb3) was inserted for subsequent 10-min periods before the beginning of the occlusion sequence (see Fig. 1). For each of the following probes (Pb4 to Pb6), coronary artery occlusion was applied sequentially for 90 s with a 60-s rest interval over a 10-min period (i.e., four occlusions were applied over the 10-min period). This constituted the coronary occlusion intervention, and a single microprobe remained in the spinal cord during this 10-min period. The probe was then replaced and the coronary occlusion cycle repeated a 2nd (probe 5) and 3rd (probe 6) time for a total duration of 30 min (Fig. 1). The average transfer replacement time between probes was 120–150 s. At the completion of experiments, the heart was removed, and the snare suture placement was visually verified.

**Spinal cord stimulation.** Preemptive, but not reactive, spinal cord stimulation has previously been shown to induce a state of cardioprotection to transient myocardial ischemia (41). To evaluate the efficacy of such stimuli to modify SP release in the thoracic spinal cord, a spring-loaded unipolar ball electrode was placed epidurally on the dorsal surface of the left C2 spinal cord segment and covered with mineral oil to prevent current spread. Through this electrode, square-wave electrical pulses were applied using a Grass constant current stimulator (Model S48) with an isolation unit (Grass Instruments, Model SIU 7) using 50-Hz, 0.2-ms duration and voltage at 90% of motor threshold. SCS was applied in two ways, reactive (P1) or preemptive (P2) (see Fig. 1). For reactive SCS, stimuli were delivered concurrently with CoAO [Fig. 1, labeled as CoAO + SCS(P1)], with sequential 90-s periods of SCS + CoAO with 60-s rest intervals between, extending over a 10-min period for each probe and repeated twice (probes Pb4–Pb6, n = 10). A time-matched SCS was done alone in another group of animals [Fig. 1, SCS (P1), n = 18]. In the second protocol, preemptive SCS was applied continuously for 45 min [Fig. 1, labeled SCS(P2), n = 9], and then in another group of rats, starting 15 min before the intermittent CoAO, and sustained for the entire duration of the coronary occlusions [Fig. 1, labeled CoAO + SCS(P2), n = 7]. As before, probes were positioned in the T4 spinal cord every 10 min, as illustrated in Fig. 1.

**Immunohistochemistry.** Ninety minutes after completion of the experimental period, animals were given a large dose of urethane (300 mg/0.5 ml) and then perfused transcardially as described before (23). The T4 spinal cord (identified by deposition of a small dot of Pontamine blue dye on the dorsal surface of the spinal cord prior to its removal) and the brain stem (near the obex) were removed and postfixed as described before (23). Consecutive transverse 40-μm sections were cut with a cryostat (IEC) at −20°C and transferred immediately to six alternate wells of polypropylene plates containing PBS.

Sections were processed for the presence of c-Fos-like-immunoreactivity (Fos-LI), as previously described (23). The localization of Fos-LI was evaluated by brightfield microscopy using an Olympus BH2 microscope. Sites in the central nervous system were identified using the atlas of Paxinos and Watson (35). Quantification of the Fos-positive cells was done by counting the number of dark brown nuclei in a 0.3 mm² area on either side of the midline for each of 10 alternate sections in the dorsal horn (corresponding to laminae I–V) at the thoracic 4 (T4) level or in a 0.6 mm² area on either side of the midline for 10 alternate sections in the brain stem at bregma −13.8 mm for each animal. In addition, c-Fos-positive neurons were also identified in the intermediolateral cell column (IML) of T4 sections in a 0.23 mm² area. Data were then grouped and averaged (±SE) for each experimental category. Sigma-stat 3.1 (Systat Software) with one-way ANOVA with post hoc comparisons (Holm-Sidak test) was used to test for differences between groups. A significance of P < 0.05 was used.

**RESULTS**

**Substance P release during cardiac ischemia or cervical spinal cord stimulation.** The summary binding patterns of the microprobes inserted into the T4 spinal cord during the CoAO protocol are presented in Fig. 2, A and B. There is a significant difference in the average grayscale levels between the 2-mm length of the in vivo rest microprobes that were in the spinal cord (i.e., 2 to 0 mm) compared with the corresponding-length section of the in vitro probes. Along this segment, the rest probes had lower average grayscale levels than the in vitro probes, indicating that endogenous irSP was released from sites in the spinal cord and displaced the radiolabeled SP from binding to the microprobes, causing the lower grayscale levels. This indicated there was a basal release of SP from the thoracic spinal cord during rest preceding cardiac ischemia. Endogenous release of SP during rest was widespread and occurred from superficial laminae I to laminae VI and VII. Note that the segment of the in vivo probes that remained outside the spinal cord (i.e., 0 to −2 mm) had virtually the same grayscale levels as the corresponding segment of the in vitro probes, and as such, there was no difference in the grayscale levels (T-values remained below the P = 0.5 significance line). Transient myocardial ischemia induced a further release of SP within the dorsal horn sites (Fig. 2B). The average grayscale levels of probes inserted into the T4 spinal cord during CoAO were lower than the grayscale levels of the rest probes along a segment corresponding to the surface of the spinal cord (i.e., 0 mm) to a depth of 0.25 mm (see Fig. 2B). This corresponds to an increased release of SP from laminae I and II in the thoracic spinal cord.
spinal cord (see Fig. 5), sites where primary sensory afferents are known to synapse (11, 13). The grayscale levels for the CoAO probes remained higher than those of the rest probes from ∼1.0 to 1.5 mm, indicating that basal levels of SP remained higher in the deeper laminae VII and X. As seen in Table 1, CoAO decreased MAP compared with rest, and this depressor effect was observed during the postocclusion recovery period. The HR was not significantly affected by cardiac ischemia. Analysis of the ECG indicated that the duration of the QRS increased during CoAO, averaging 29.7 ± 3.8 ms compared with 16.8 ± 2.3 ms during rest control preceding the occlusions (P = 0.01). In addition, ST-segment elevations were consistently observed during occlusions and returned back to baseline during recovery periods.

Spinal cord stimulation by itself did not alter the pattern of SP release at the T4 spinal level from basal levels, either when delivered intermittently for 30 min [Fig. 2C, SCS(P1)] or continuously for 45 min [Fig. 2D, SCS(P2)]. Moreover, neither continuous nor intermittent SCS altered MAP or HR compared with resting levels (see Table 1), nor did it affect the depressor effect of CoAO when applied simultaneously with the occlusions (Table 1). However, in contrast to CoAO alone, coronary occlusion applied during continuous SCS was associated with a bradycardia that was maintained into the recovery phase (Table 1).

### Substance P release during cardiac ischemia and cervical spinal cord stimulation

High cervical spinal cord stimulation alters the pattern and distribution of myocardial ischemia-induced substance P release within the thoracic spinal. The grayscale levels of the probes during intermittent SCS applied simultaneously with the occlusion (see Fig. 3A) were lower than the grayscale levels of the probes during coronary occlusion alone along the segment from 0.6 mm to 2 mm, indicating this form of spinal cord stimulation caused greater SP release.

### Table 1. Cardiovascular responses during coronary occlusion and spinal cord stimulation

<table>
<thead>
<tr>
<th>Procedure</th>
<th>n</th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
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<tbody>
<tr>
<td><strong>MAP</strong></td>
<td></td>
<td>Rest</td>
<td>Procedure</td>
</tr>
<tr>
<td>CoAO</td>
<td>10</td>
<td>87±4</td>
<td>71±4*</td>
</tr>
<tr>
<td>SCS (P1)</td>
<td>18</td>
<td>79±6</td>
<td>72±6*</td>
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<tr>
<td>SCS (P2)</td>
<td>9</td>
<td>87±3</td>
<td>84±3</td>
</tr>
<tr>
<td>SCS (P1) + CoAO</td>
<td>10</td>
<td>91±4</td>
<td>81±6*</td>
</tr>
<tr>
<td>SCS (P2) + CoAO</td>
<td>7</td>
<td>76±3</td>
<td>63±4*</td>
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Values are expressed as means ± SE; n, number of rats in group. MAP, mean arterial pressure; HR, heart rate; CoAO, coronary artery occlusion of the left anterior descending artery; SCS, spinal cord stimulation; P1, protocol 1 (intermittent stimulation, 90 s on, 60 s off for 10 min); P2, protocol 2 (continuous stimulation, 45 min); SCS + CoAO, coronary occlusion with stimulation. *P ≤ 0.05 from rest; #P ≤ 0.05 from corresponding CoAO group.
from deeper laminae compared with cardiac ischemia alone. In contrast, the grayscale levels of probes inserted into the T4 spinal cord during continuous spinal cord stimulation and coronary occlusion (see Fig. 3B) were greater than the grayscale levels of CoAO probes along the segment of the probes from 0 mm to 0.5 mm, indicating this form of stimulation increased the release of SP from laminae I–VII (see Fig. 5). For deeper laminae, preemptive and continuous SCS did not alter the pattern of SP from that induced by CoAO alone (Fig. 3B).

The effect of the two different forms of spinal cord stimulation on the SP release induced by occlusive activation of cardiac ischemia-sensitive afferent fibers is further illustrated in Fig. 4. The average grayscale levels for probes during simultaneous intermittent SCS (P1) and CoAO (Fig. 4A) were lower than the intermittent SCS (P1) alone, indicating that CoAO itself caused a greater release of SP from laminae I–VII and that reactive SCS was not effective in mitigating the input signal from cardiac sensory nerves at the thoracic spinal level. In contrast, continuous SCS applied prior to and during CoAO [SCS (P2)+CoAO] resulted in similar grayscale levels to the probes during SCS (P2) alone (Fig. 4B), indicating the effects of the occlusion on SP release were suppressed by preemptive SCS. The difference in the effects of preemptive, continuous SCS compared with intermittent and reactive SCS can be seen in Fig. 4C. Here, the grayscale levels of the probes during preemptive, continuous SCS [SCS (P2)+CoAO] were significantly greater than those of the probes during intermittent stimulation [SCS (P1)+CoAO]. This clearly shows the suppression of CoAO-induced SP release along the entire 2-mm length of probes inserted into the thoracic spinal cord.

**Effect of cervical spinal cord stimulation on thoracic spinal and nucleus tractus solitarius neuronal activation.** Transient coronary occlusion, with and without concurrent SCS, altered the activities of brain stem and spinal cord neurons associated with the neural control of the heart, as indicated by number of c-Fos-positive neurons at the T4 spinal level (Fig. 5 and 6, A and C) and brain stem (Fig. 6B). Application of cervical SCS by itself, either intermittently [SCS(P1)] or continuously [SCS(P2)], did not significantly change the number of c-Fos-positive neurons in laminae I–V in the T4 thoracic spinal cord, compared with the sham control animals (Fig. 6A). However, cardiac ischemia induced by left anterior descending coronary artery occlusion (CoAO) did increase c-Fos expression in these same spinal regions (T4, laminae I–V) (Fig. 6A). While intermittent (reactive) SCS did not alter the CoAO-induced increase in these same spinal segments, preemptive SCS [SCS (P2)] significantly reduced the number of c-Fos-positive neurons in T4 laminae I–V (Fig. 6A). A similar pattern was noted bilaterally in the nucleus tractus solitarius (NTS) (Fig. 6B), with preemptive SCS being effective in reducing the degree of neural activation associated with transient coronary occlusion. Neurons contained with the IML region at the T4 spinal level showed a different pattern. While coronary occlusion by itself induced little change in c-Fos expression, preemptive SCS increased the number of c-Fos-positive neurons, both when applied in basal conditions and during coronary artery occlusion (Fig. 6C).

**DISCUSSION**

This study focuses on the changes in substance P release at the spinal level that are associated with the nociceptive signals coming from the ischemic heart in response to high cervical neuromodulation. There are three primary findings from this study: the first is that cardiac ischemia increases the release of SP from the superficial laminae in the thoracic spinal cord, confirming the results from our previous studies (22, 24); the second is that whereas preemptive, continuous cervical SCS suppresses release of the putative nociceptive neuropeptide, SP, during cardiac ischemia, intermittent and reactive SCS did not; and the third is that whereas preemptive, continuous cervical SCS suppressed myocardial ischemia-induced activation of thoracic and brain stem/NTS neurons, intermittent and reactive SCS did not. Although the impact of antianginal (15, 18–20, 30–32) and cardioprotective (3, 7, 8, 40, 41) effects of spinal cord stimulation are becoming increasingly appreciated, little is known about the neurochemical basis of such neuro-
modulation at principal synaptic processing sites (peripheral and central) of the cardiac nervous system and at the heart itself. The principal findings from this study provide mechanistic insights into the modulation of the cardiac ischemic neurochemical signal by spinal cord stimulation with effects manifest in spinal and supraspinal processing sites associated with reflex responses to activation of cardiac ischemic sensitive afferents.

SP in the thoracic spinal cord dorsal horn is a principal neuropeptide mediator of the signal transmission of myocardial ischemia and nociception conveyed via the sympathetic CISAN (13, 26). The cell bodies of sympathetic CISAN are found in dorsal root ganglia of spinal segments C8–T9 (cervical 8-thoracic 9), with the majority associated with T2–T6 spinal segments (28). The primary central input from these CISAN occurs into spinal cord laminae I–V, VII, and X, subsequently exciting cells in the spinothalamic tract (T1–T6 and C1 and C2 levels), as well as other ascending pathways (10, 16, 37). We previously showed that transient occlusion of the rat left anterior descending coronary artery increased the release of SP from the superficial (I and II) and deeper (III–VII) dorsal laminae (24). This increased release of SP was associated with the activation of CISAN because this profile was maintained only for the time when the coronary occlusion was applied and was eliminated during transient occlusion following thoracic dorsal rhizotomy (24).

Two forms of electromodulation therapy are used to treat intractable angina: left vagal stimulation and spinal cord stimulation, at either high cervical (C1–C2) or thoracic (T1–T2) segments (2, 29, 31, 38–39, 44). The antianginal effect of SCS most likely does not reflect a direct conduction block of spinothalamic tract neurons (34) since stimulatory parameters would not directly activate thin, high-threshold fibers or directly activate descending inhibitory pain pathways (29) and since patients still perceive pain at critical levels of myocardial ischemia (6, 8, 29). Understanding the differences between reactive vs. preemptive electroneuromodulation may be a function of what neural pathways are activated by such procedures.

Transient ischemia activates both sympathetic and vagal CISAN (25). Whereas, sympathetic CISAN input directly to the high thoracic cord, modulating neurons projecting to cervical and supraspinal sites (10, 16, 37), vagal CISAN cell bodies are found in nodose ganglion and project to dorsal laminae in C1–C3 (17), as well as centrally via the 10th cranial nerve to the medullary neurons in the nucleus tractus solitarius (5). Descending projection from the nucleus tractus solitarius to the high cervical area, in turn, has been implicated in modulation of SST projections activated by cardiac nociceptive afferents (16, 17). As such, spinal cord stimulation, like vagal afferent stimulation, most likely involves propriospinal neuronal interactions within the spinal neural hierarchy with the higher cervical signal segments (C1–C2) serving a primary integrative function and modulating the nociceptive processing of neurons in the higher thoracic spinal segments (9, 17).
C2 stimulation modulates SP release from cardiac afferents

Fig. 6. A: quantification of c-Fos-positive neurons in T4 spinal segment. c-Fos-positive cells were counted under bright field microscopy within laminae I–V for the left and right sides of spinal segments, averaged and expressed as a function of the cross-sectional area measured (means ± SE). B: quantification of c-Fos-positive neurons (right and left average) in the nucleus tractus solitarius (NTS) at brain stem level bregma –13.8mm. C: quantification of c-Fos-positive neurons (right and left average) in each of these central nervous system regions in sham time controls (solid bar), during sole SCS (hatched bar; intermittent, P1 or continuous, P2 SCS), transient coronary artery occlusion (horizontally lined bar), or combined SCS (P1 vs. P2) and transient coronary artery occlusion (cross-hatched bar). *P < 0.05 vs. sham control; + P < 0.05 CoAO vs. CoAO+SCS (P1 or P2).

previous data demonstrated that intermittent reactive vagal afferent stimulation caused a change in the SP release profile and suppressed its release from superficial laminae (22). Our current study indicates that while the analogous reactive stimulation protocol using high cervical SCS was ineffective in modulating such SP release, preemptive SCS was. Our finding that continuous and preemptive SCS decreases SP release from superficial laminae in the T4 spinal cord is important since other studies have shown that the majority of the primary synapses from CISAN (C-fiber type) occur within laminae I and III (16, 28). Thus SCS modulation of the CISAN input signal may reflect, in part, an inhibition of SP release from the sympathetic afferent neuron projections. The reduced SP release from CISAN fibers during myocardial ischemia and decreased c-Fos expression in thoracic dorsal horn and NTS expression subsequent to preemptive SCS may also be reflective of a SCS-mediated effect rendering myocytes ischemic resistant (41) and/or a stabilization of peripheral autonomic reflex activation during ischemic episodes (3, 4, 17, 18), thereby decreasing the relative stress levels associated with the transient myocardial ischemia. Future studies will be required to distinguish between these myocyte vs. neural contributions to the antianginal and cardioprotective effects of neuromodulation therapy.

Our results are also relevant to the findings that preemptive, but not reactive SCS, was effective in reducing infarct size during short-term myocardial ischemia (41). Neither 30 min nor 3 h of reactive SCS was able to reduce infarct size, while preemptive SCS that was applied 15 min before ischemia and was sustained for a total of 45 min did decrease infarct size. Our preemptive protocol was identical to theirs. Southerland et al. (41) also showed there was both a frequency (50 Hz) and duration (at least 5 min) threshold for SCS to be effective in reducing infarct size. These findings together with our results, suggest that the absolute stimulation time is not as important as the timing of the stimulation.

Neither intermittent nor continuous SCS alone applied at the C2 level inhibited basal SP release from thoracic dorsal horn nor did they change c-Fos expression in the thoracic spinal cord (laminae I–V) or NTS. This is unlike the effect of left vagal afferent stimulation, which increased neuronal activation in the NTS (22). Left vagal afferent nerve stimulation intervenes at the upper cervical spinal cord and brain stem (17) and excites inhibitory descending pathways originating within the NTS (16), which, in turn, most likely excites inhibitory priospinal pathways in the cervical level to modify the input signals from the heart at the thoracic spinal level (17). Our c-Fos results support the hypothesis that the antinociceptive effects of SCS do not require such a supraspinal pathway (29, 31), since there was no increase in neuronal activation in NTS sites. Our data also indicate that high cervical SCS, by itself, does not directly activate thoracic dorsal horn sites. Our results are in contrast to a previous study, where SCS resulted in higher levels of c-Fos expression in the thoracic spinal cord (segments 2 and 3) (12). This is not surprising since in that study (12), SCS was applied at a lower spinal level (C7–T2) at higher electrical parameters, and the increased c-Fos expression was most likely the result of the direct stimulation of the thoracic spinal segment. Our data suggest that short-term high cervical SCS exerts minor influences on basal spinal cord or brain stem neural function, yet in conjunction with data from the dog (18) and rabbit (40, 41) induces a state where there is increased tolerance to subsequent ischemic challenges.

Against the stress of transient myocardial ischemia, preemptive and reactive SCS induced different effects upon SP release in the T4 dorsal columns and neuronal activities in the T4 spinal segment and within the NTS brain stem region. In contrast to the suppressing effects of preemptive SCS on T4 SP release, intermittent and reactive SCS actually increased the release of SP from thoracic laminae IV–VII. While direct recordings from peripheral intrinsic cardiac neurons demonstrate similar levels of suppression of peripheral autonomic function regardless of preemptive vs. reactive SCS application (18), only preemptive and not reactive SCS was effective in reducing infarct size to transient myocardial ischemia (41). In addition, it is possible that the intermittent application of SCS was subthreshold for mediating the effects on spinal cord SP release. In this regard, while peripheral autonomic reflex func-
tion demonstrates long-term “memory” to SCS (6), the suppressive effects on evoked activity of spinothalamic tract cell neurons was evident only during SCS (18).

In contradistinction to the effects of preemptive SCS on neuronal activities in the T4 dorsal horn and within the NTS, continuous, preemptive SCS stimulation increased c-Fos expression in the T4 IML region by itself and augmented the active cells with the subsequent ischemic stress. Preemptive, but not reactive, SCS reduces myocyte cell death due to transient myocardial ischemia (40, 41), an effect dependent on activation of cardiac adrenergic neurons (41). Thus, preemptive SCS may recruit an additional population of sympathetic preganglionic neurons that, in turn, induce a state of cardioprotection to subsequent ischemic stress. At the same time, SCS exerts a second influence of peripheral autonomic function, restraining the excitatory reflex responses to transient myocardial ischemia (18), an effect that is maintained for at least 45 min after SCS termination (6).

Perspectives and Significance

Information from these experiments provides insight into the neurochemical changes that occur in the spinal cord during cardiac ischemia. Together with findings from previous studies (22, 24), SP is identified as a mediator of the cardiac ischemic signal. Furthermore, these data indicate that preemptive, but not reactive, SCS is effective in inhibiting SP release during ischemia, and support the findings that this inhibition is mediated via cervical propriospinal pathways. This adds to our understanding of the beneficial effects of clinical neuromodulation therapy being used for individuals with chronic, intratable angina and reinforces the suggestion that either cervical or thoracic spinal cord stimulation are effective treatment options. This study is an important adjunct to findings showing that preemptive, but not reactive, neuromodulation therapy is effective in reducing infarct size following transient ischemia (41). The beneficial effects of preemptive SCS are based on modifying the ischemic afferent processing not only within the central nervous system, but also by the intrinsic cardiac nervous system, and as such, may exert a stabilizing effect on the latter (41). Given that inhibitory propriospiunal pathways are involved with the modulation of the cardiac ischemic signal, it will be of great interest to determine whether this effect is mediated by the release of inhibitory substances, such as opiates, which appear to be the case with other forms of electroneuromodulation (22).

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