c-Fos induction in spinal cord neurons after renal arterial or venous occlusion

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Rosas-Arellano, M. Patricia, L. Pastor Solano-Flores, and John Ciriello. c-Fos induction in spinal cord neurons after renal arterial or venous occlusion. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R120–R127, 1999.—Experiments were done in the anesthetized rat to identify the dorsal root ganglia (DRG) and the spinal cord segments that contain neurons activated by either renal venous occlusion (RVO) or by renal arterial occlusion (RAO). Fos induction, detected immunohistochemically in DRG and the spinal cord neurons, was used as a marker for neuronal activation. RVO induced Fos immunoreactivity in neurons in the DRG of spinal segments T5–L2 on the side ipsilateral to that of occlusion. The largest number of Fos-labeled neurons was found in the T11 DRG. In the spinal cord the largest number of Fos-labeled neurons was found in the ipsilateral dorsal horn of spinal segments T13–T2, predominantly in a cluster near the dorsomedial edge of laminae I–II. A few additional Fos-labeled neurons were observed in laminae IV and V. After RAO Fos-labeled neurons were found in the ipsilateral DRG of spinal segments similar to those observed to contain neurons after RVO. However, most of the Fos-labeled neurons were observed within the T12–L1 DRG. In the spinal cord Fos-labeled neurons were scattered throughout lamina I–II of the ipsilateral dorsal horn of spinal segments T5–L2, although the largest number was observed at the T13 level. Additionally, a distinct cluster of Fos-labeled neurons was observed predominantly in the region of the ipsilateral intermediolateral cell column, although a few neurons were found scattered throughout the nucleus intercalatus, central autonomic areas, and laminae IV and V of the cord bilaterally. No Fos labeling was observed in the complementary contralateral DRG or dorsal horns after either RVO or RAO. In addition, renal nerve transaction prevented Fos labeling in the ipsilateral DRG and dorsal horns after RVO or RAO. Taken together, these data suggest that functionally different renal afferent fibers activate DRG neurons that may have distinct projections in the spinal cord.

afferent renal nerve; kidney; renal receptors; renal blood flow; cardiovascular regulation

EXPERIMENTAL EVIDENCE is available that suggests that sensory information from the kidney may be of considerable importance in renorenal sympathetic reflexes, the regulation of the circulation, and in the pathogenesis of hypertension in certain experimental models of the disease (5, 9, 20, 21, 24, 34, 44, 45, 49). Afferent information from the kidney carried in afferent renal nerves (ARN) is thought to originate in several different classes of sensory receptors: renal mechanoreceptors that are sensitive to changes in arterial, venous or ureteral pressure, or mechanical stimuli (1, 3, 31, 32, 42, 47), chemoreceptors that are activated by ischemia and changes in the ionic environment of the interstitium (30, 37–39), and possibly nociceptors (10). Selective activation of renal receptors (10, 37–39) and electrical stimulation of ARN (5–7, 35, 41) have been shown to elicit a variety of hemodynamic responses mediated by the sympathetic nervous system and humoral mechanisms.

ARN information has been shown to influence the activity of neurons at several different levels of the neuraxis (reviewed in Ref. 43). Although it has been suggested that renal sensory information may reach medullary and forebrain sites through the vagus nerves (17), it is generally accepted that most of the renal afferent information is carried by ARN to the spinal cord (8, 12, 26). ARN are composed mostly of small nonmyelinated (C-type) fibers or thin myelinated Aδ-fibers (2). These fiber types have been associated with conveying specific afferent renal information to the spinal cord (3, 31, 32, 38, 47). ARN fibers have been shown to enter the ipsilateral dorsal horn through dorsal root ganglia (DRG) T6–L2 (8, 12, 15, 26). However, the location of the DRG that carry ARN information from functionally different renal receptors to the central nervous system has not been identified. In addition, the distribution of neurons in the spinal cord that receive specific ARN information has not been systematically investigated.

The present study was done to determine the spinal level of DRG that relays ARN information to the spinal cord evoked by renal venous (RVO) or renal arterial (RAO) occlusion. In addition, the location and distribution of neurons in the spinal cord activated by RVO or RAO were investigated. Increased intrarenal pressure following RVO has been shown to activate renal chemoreceptors (31, 32, 47), whereas RAO is thought to activate renal chemoreceptors by the resulting ischemia (29, 37, 38). Activated neurons in DRG and the spinal cord were immunohistochemically identified as the result of the induction of the intermediate early gene c-fos. c-Fos has been implicated as one of the third messengers in intracellular signal transduction pathways in a variety of cells (27, 28). In the nervous system, the basal expression of c-Fos is relatively low, but it can be induced rapidly and transiently by growth factors, neurotransmitters, second messengers, or membrane depolarization (14, 27, 28, 40). The c-Fos immunohistochemical technique has been used for the detection of changes in neuronal activity during the application of a variety of specific physiological stimuli (4, 13, 16, 18, 19, 33, 40), including electrical stimulation of ARN (43).

METHODS

Isolation of renal artery and vein. Experiments were done in 22 male Wistar rats weighing 250–350 g under thiobutabarbital anesthesia (Inactin, 100 mg/kg ip). The right femoral
artery was cannulated with PE-50 tubing for the recording of arterial pressure (AP). AP was recorded through a Statham transducer (model P23X4) and continuously monitored on a Grass model 79E polygraph. A lateral laparotomy was performed to expose the left kidney. Under a stereoscopic dissecting microscope, the left renal artery and vein were identified as they emerged from the hilus of the kidney, cleaned of connective and fat tissue, and separated from the renal nerves using blunt dissection with glass rods. A silk thread (Ethicon A-52, 3–0) was then passed around either the renal artery or vein taking care not to damage the blood vessels or renal nerves. During the surgical procedure the exposed tissue was covered with saline-soaked gauze, and isotonic saline was constantly applied to the surgical area to prevent the drying of the tissues. Body temperature was maintained at 37°C using a heating pad controlled by a Yellow Springs temperature controller.

After the surgical procedures, the animals were allowed to stabilize for a period of 90 min before the occlusion of either the renal artery (n = 6) or the renal vein (n = 6), or during sham occlusion (n = 4). The renal artery or vein was occluded for a period of 30 or 90 min by gently tightening the silk thread around the blood vessel. Sham occlusion consisted of moving the thread, without tightening it, around one of the renal blood vessels. In animals in which the renal vessels were occluded for only 30 min, the ligature was carefully removed and the animals were allowed to survive for an additional 60 min. In four additional animals, after isolation of the renal blood vessels and nerves, the renal nerves were transected and the animals were allowed to stabilize for at least 2 h before occlusion of the renal artery (n = 2) or renal vein (n = 2). One animal from each group was occluded for either the 30- or 90-min period. Occlusion of the renal artery would be expected to cause the activation of the renin-angiotensin II system and elicit an associated transient increase in AP. Therefore, the effect of administering in the drinking water of the animals (for at least 2 days before the experiment) the angiotensin-converting enzyme inhibitor enalapril maleate (20 mg·kg⁻¹·day⁻¹, Sigma Chemical, St. Louis, MO) on the Fos labeling in DRG and the dorsal horns was determined in two additional animals after RAO.

Immunohistochemical procedures. After the 90-min period from the beginning of occlusion or sham occlusion of the renal vessels, the animals were perfused transcardially with 400 ml of 0.9% cold saline followed by 400 ml of 4% paraformaldehyde in 0.4 M phosphate buffer (PBS, pH 7.2) at 4°C. The spinal cord at the Th12–L2 levels and the corresponding DRG regions were removed and stored for 4–6 h in the NH2-terminal epitope, at a dilution of 1:5,000 in normal goat serum (Vector Laboratories, Burlingame, CA) that had been diluted at 1:100 with PBS containing 0.3% Triton X-100 at 4°C (19, 43). After 72 h the sections were washed in PBS and placed in goat biotinylated anti-rabbit IgG (Vectastain) diluted 1:200 in PBS 0.3% Triton X-100 for 30 min. Sections were rinsed, placed in solution of methanol and hydrogen peroxide for 30 min, washed in PBS, and then placed in an avidin-biotin complex reagent (Vectastain) in PBS to visualize the peroxidase reaction product. The sections were then washed, mounted onto glass slides, dried, placed into an acid-alcohol solution, and placed under a coverslip with glycerol-PBS.

Controls for Fos immunoreactivity included processing sections either without the primary antibodies or after preadsorbing the primary antisera with the antigen (19, 43). No immunoreactivity was found in DRG or the spinal cord in each case.

Data analysis. Spinal cord and DRG sections were analyzed and photographed under bright-field microscopy (Leitz Diaplan microscope). The location of Fos-labeled neurons was mapped on projection drawings of the DRG and spinal cord for each animal. The total number of Fos-labeled (>200 magnification) neurons in each of the DRG and the total number of Fos-labeled neurons in 10 consecutive sections of each of the spinal cord segments were counted by at least two independent investigators. The value for the spinal cord segments was expressed as the average number of Fos-labeled neurons per section. The stereotaxic atlas of the rat brain of Paxinos and Watson (36) was used to identify spinal cord regions. All values were expressed as means ± SE. Statistical analysis was done using a one-way ANOVA followed by the Bonferroni post hoc test when the ANOVA indicated a statistical significance (P < 0.01). A value of P < 0.01 was used to indicate statistical significance.

RESULTS

As summarized in Table 1 AP in the sham-occluded animals did not change during the 180 min of the experiment. Similarly, the level of AP in either the RVO or RAO animals was not significantly altered after occlusion compared with precocclusion levels. In addition, AP was found not to be altered in any of the animals in which renal nerves were transected or in which the animals had been treated before the renal occlusion with the angiotensin II-converting enzyme inhibitor.

Figure 1 summarizes the distribution of Fos-labeled neurons in DRG, ipsilateral to the side of stimulation, following either RVO or RAO. No labeled neurons were observed in the corresponding contralateral DRG in either the RVO or RAO animals. In addition, no Fos labeling was found in either the ipsilateral or contralateral DRG at similar levels in the sham-occluded animals. Furthermore, in animals in which the ipsilateral renal nerves were transected before either RVO or RAO no Fos-labeled neurons were observed in the DRG or dorsal horns. The largest number of Fos-labeled neurons in the DRG of the RVO animals was found at the Th12 level (Figs. 1 and 2A). Approximately 40–70% more Fos-
labeled neurons were observed in the DRG at this level compared with other DRG in RVO animals. At the T11 level the number of Fos-labeled neurons resulting after RVO was greater than that from RAO. On the other hand, the largest number of Fos-labeled neurons in the RAO animals was found in DRG T13 (Figs. 1 and 2B). At the T12-L1 DRG levels, the number of Fos-labeled neurons after RAO was greater than that after RVO (Fig. 1). In general, a larger number of DRG neurons was Fos-labeled following RAO compared with RVO (Fig. 1).

Within the spinal cord Fos labeling was observed in the ipsilateral dorsal horns in both the RVO (Fig. 3) and RAO (Fig. 4) animals throughout the T8-L2 spinal cord segments (Figs. 5 and 6). No Fos-labeled neurons were found in either the contralateral dorsal horns (Figs. 3A and 4A) or in either the ipsilateral and/or contralateral dorsal horns of the sham-occluded animals. Figure 5 schematically illustrates the general distribution pattern of Fos labeling in transverse sections of the thoracic cord from animals following RVO or RAO. This labeling pattern was representative of all cases within each of the RVO or RAO groups of animals, regardless of the time the renal artery or vein was occluded (30 or 90 min).

Several similarities and differences in the distribution of Fos-labeled cell neurons were observed in the spinal cord of RVO and RAO animals (Figs. 3–6). In both RVO and RAO animals the largest number of Fos-labeled neurons was found in segments T11-T13. The density of Fos-labeled neurons in both the RVO and RAO animals was observed to be higher in the dorsomedial aspect of lamina I-II at the T11-T12 level and at T13 in the RAO animals (Figs. 5 and 6). However, in the RVO animals the Fos-labeled neurons in the dorsomedial aspect of the dorsal horn were within a well-defined cluster at the T11-T12 levels (Figs. 3 and 5), but was absent in the T13-L2 segments or the upper thoracic segments.

The RAO animals were found to contain a significantly larger number of Fos-labeled neurons in lamina I along the dorsolateral aspect of the dorsal horn at all spinal segments compared with the RVO animals (Figs. 3–6). Additionally, the RAO animals exhibited a peak in the number of Fos-labeled neurons at the T13 level in this dorsolateral region (Figs. 4C and 6). This contrasted with the finding in the RVO animals of an almost constant number of Fos-labeled neurons in this dorsolateral aspect of lamina I throughout the T8-L2 levels (Fig. 6). It was also observed in the RAO animals (Fig. 4, B and C) that the Fos-labeled nuclei of most neurons in lamina I of the dorsolateral horn (Fig. 6), just ventral to the tract of Lissauer, were larger than those observed in RVO animals (Fig. 3C). These large labeled nuclei, observed only in the RAO animals, may belong to neurons that form the large marginal cells of Waldeyer (46).
In both the RAO and RVO animals, a few Fos-labeled neurons were found scattered throughout laminae IV and V bilaterally. In addition, in both groups of experimental animals, the region near the medial aspect of the intermediomedial nucleus contained a few Fos-labeled neurons. It was apparent that the number of Fos-labeled neurons in this region was greatest in the RVO animals throughout the T8-L1 levels. In the RAO animals only the T13 spinal segment contained a few labeled neurons in this region, just medial to the intermediolateral nucleus (Fig. 5). In the RAO animals a cluster of Fos-labeled neurons was also observed within the intermediolateral cell column (Figs. 5 and 7A) and a few were found scattered throughout the nucleus intercalatus (Figs. 5 and 7A) and in the region lateral and dorsolateral to the central canal, an area referred to as the central autonomic region (Figs. 5 and 7A) of the thoracolumbar cord. In contrast, in the RVO animals a few scattered neurons were observed in the nucleus intercalatus and central autonomic region, and few, if any, neurons contained Fos labeling in the intermediolateral nucleus (Figs. 5 and 7B).

Because RAO would be expected to activate the renin-angiotensin II system, the possibility existed

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Fig. 3. Bright-field photomicrographs of transverse sections of T11 (A and B) and T12 (C) spinal cord segments show Fos-labeled neurons in ipsilateral dorsal horn after RVO. Note dense cluster of Fos-labeled neurons in dorsomedial aspect of laminae I-II, just ventral to dorsal columns. In addition, note lack of Fos-labeled neurons in contralateral dorsal horn. Box in A is photomicrograph in B. DC, dorsal columns; dlf, dorsolateral funiculus; LT, tract of Lissauer; I, II, III, IV, X, laminae of spinal cord. Calibration marks equal 100 µm.

Fig. 4. Bright-field photomicrographs of transverse sections of T12 (A and B) and T13 (C) spinal cord segments show Fos-labeled neurons in ipsilateral dorsal horn after RAO. Note Fos-labeled neurons throughout lamina I. In addition, although no direct measurement of labeled Fos nucleus was made, it is apparent in B and C that those labeled neurons under tract of Lissauer in dorsolateral aspect of lamina I are larger than those found in dorsomedial aspect of laminae I-II and appear to form distinct group of Fos-labeled neurons. Finally, note lack of Fos labeling in contralateral dorsal horn. Box in A is photomicrograph in B. Open arrow in A shows Fos-labeled neurons in intermediolateral cell column. Abbreviations are defined in legend for Fig. 3. Calibration marks equal 100 µm.
that the increased circulating levels of angiotensin II may have contributed to the Fos labeling observed in either the DRG or dorsal horns. In the animals given an angiotensin II-converting enzyme inhibitor, no differences were observed in the distribution of Fos-labeled neurons in either the DRG or the dorsal horns after RAO.

**DISCUSSION**

The c-Fos immunohistochemical method has been proposed as a method for the detection of changes in neuronal activity during the application of different physiological stimuli (13, 14, 16, 27, 28, 40). Recently, this method has been used to identify the neural pathways associated with cardiovascular and body fluid regulation (18, 19, 33). In addition, this method has been used to identify the brain stem and forebrain sites activated by electrical stimulation of ARN (43). In this study some of the neuronal pathways that may mediate mechanoreceptor and/or chemoreceptor afferent information from the kidney were identified following RAO or RVO.
It may be argued that the stimuli applied or the surgical stress induced nonspecific Fos expression in DRG and in the spinal cord. This possibility is unlikely as both the DRG and dorsal horn of the side contralateral to the application of the stimuli did not contain Fos-labeled neurons. In addition, the Fos labeling observed in the ipsilateral DRG and spinal cord was not due to the surgical stress as no Fos-labeled neurons were observed in sham-occluded animals that underwent the same surgical procedures, but the blood vessels were not occluded. Furthermore, denervation of the ipsilateral kidney before either RAO or RVO eliminated the Fos labeling observed in the DRG and dorsal horn.

It may also be suggested that changes in AP following RAO or RVO may have contributed to the induction of c-Fos, possibly as a result of the reflex activation of the baroreceptor reflex by the AP changes. However, this possibility is also unlikely as no significant changes in AP resulted after RAO or RVO in this study. In addition, in animals in which the transient rise in AP resulting from the activation of the renin-angiotensin II system after RAO was prevented by the prior administration of an angiotensin II-converting enzyme inhibitor, the pattern of Fos labeling in both the DRG and dorsal horn was not altered after RAO. This observation suggests that the Fos labeling was not due to an increase in AP nor to the actions of angiotensin II directly on DRG or dorsal horn neurons, or on central pathways that may alter inputs to dorsal horn neurons. Furthermore, if the small transient decrease in AP that occurred after RVO contributed to the expression of Fos in the spinal cord as a result of the reflex activation of baroreceptors, Fos-labeled neurons would be expected within the intermediolateral cell column of the thoracolumbar cord. This would occur as a result of the reflex activation of preganglionic sympathetic neurons to increase vasomotor tone. However, no Fos-labeled neurons were found in this region of the spinal cord.

It has been reported that application of a prolonged stimulus to a sensory system is required to adequately demonstrate Fos labeling in central structures (4, 13, 14, 27, 28, 40). In this study, the possibility cannot be eliminated that the prolonged application of the stimulus altered renal glomerular pressures in the stimulated kidney as a result of the changes in renal AP and may have induced some damage to the kidney. No measurements of these variables were made in this study as the procedures used to assess these variables themselves may have induced nonspecific Fos labeling. However, the finding that the patterns of Fos labeling were similar after either period of stimulus application and that a differential pattern of Fos labeling resulted after RAO or RVO suggests that these renal changes, although likely present, were probably not responsible for the observed Fos labeling.

The finding that DRG of the lower thoracic spinal cord contained the somata of ARN was not unexpected. The location of ARN somata has previously been shown using the anterograde transport of horseradish peroxidase, after the application of horseradish peroxidase to ARN or after its injection into the kidney (8, 12, 15, 17, 26, 48). In addition, the finding that the contralateral DRG did not contain Fos-labeled neurons is consistent with the findings of the lack of labeled neurons in the contralateral DRG after application of retrograde tracers to ARN (8). However, the finding in this study of DRG neurons that may carry functionally different sensory modalities from the kidney has not been previously described. This finding in the present study suggests the possibility that afferent fibers from renal receptors activated by RVO or RAO share common
DRG pathways but also maintain distinct preferential routes for entry into the spinal cord. Most of the DRG neurons activated by RAO were found at the T12–T13 level, whereas most of the DRG neurons activated following RVO were found at the T11–T12 level. These differences were also maintained in the dorsal horn. The functional significance of this anatomic separation in the spinal cord is not clear. However, it is not unreasonable to suggest that it may represent the location of neurons that relay functionally specific renal sensory information to varying medullary or forebrain sites.

Distinct groups of Fos-labeled neurons, presumably second-order neurons in ARN pathways, activated by RAO were found in the dorsolateral aspects of lamina I of spinal segments T13, whereas after RVO they were found in the medial aspect of laminae I-II of spinal segments T11. The finding of labeled neurons in laminae I-II of the ipsilateral spinal cord is consistent with the earlier demonstration of horseradish peroxidase-labeled ARN fibers in similar regions of the spinal cord following injections of the tracer into the hilus region of the kidney or the application of the tracer to ARN (8, 26). In addition, their distribution is consistent with electrophysiological studies showing neurons activated by ARN stimulation or to selective activation of renal receptors in the rat (23, 24). Neurons located within these layers of the spinal cord have been shown to relay this renal afferent information to brain stem and forebrain areas (reviewed in Ref. 43). Similar areas in the spinal cord have been reported to contain Fos-labeled neurons following electrical stimulation of ARN (43). However, it is of interest to note that electrical stimulation of ARN has been shown to induce Fos labeling in neurons predominantly outside of lamina I (43). Although the reason for this discrepancy is not known, it is possible that the parameters of stimulation used in the previous study (43) may have preferentially or predominantly activated ARN fibers carrying one type of sensory modality. From the data obtained in this study, it may be suggested that the electrical stimulus (43) may have activated fibers that respond to RVO, thought to be primarily of the small myelinated type (45). The finding that RAO activated preferentially large neurons in the dorsolateral aspect of the dorsal horn is consistent with this suggestion, as this region of the dorsal horn is known to receive a dense innervation from nonmyelinated afferent fibers containing substance P. ARN fibers have been shown to contain substance P (22). These large neurons may be components of spinothalamic or spinohypothalamic pathways relaying renal sensory afferent information directly to forebrain structures without intervening synapses at medullary levels (9). The finding that activation of renal receptors by RAO or RVO induced Fos activity in the regions of the central autonomic area, lamina X, and the intermediolateral cell column bilaterally is consistent with the previous report that electrical stimulation ARN induces Fos expression in these spinal cord regions (43). This observation is supported by the finding that activation of renal receptors evoke renorenal reflexes (11, 25).

**Perspectives**

This study has provided a functional mapping of the segmental distribution of ARN activated by changes in renal blood flow. The DRG and spinal cord neurons activated by the occlusion of the renal artery or vein likely represent neurons responding to the activation of functionally different renal receptors, such as renal chemoreceptors and/or mechanoreceptors (for reviews see Refs. 44 and 45). This suggestion is based on the earlier demonstration that R1 and R2 renal chemoreceptors have been shown to be selectively activated by renal ischemia as a result of RAO (37–39). Prolonged renal ischemia has been shown to produce an elevated discharge from renal chemoreceptors, even up to 30 min after death (38). In addition, these chemoreceptors have been demonstrated not to respond to changes in intrarenal pressure. On the other hand, an increase in intrarenal pressure after RVO has been shown to be an effective stimulus for the activation of renal mechanoreceptors (31, 32, 47) and to evoke an increase in renal nerve activity (3, 31, 32, 47). Decreasing renal AP by RAO has been reported to reduce and even abolish neural activity originating from renal mechanoreceptors (3, 31, 32, 47). However, the possibility cannot be completely excluded that both types of stimuli may have activated both sets of renal receptors. Prolonged RVO may have produced an ischemic response as a result of venous stasis. This may account for some of the overlap in the distribution of Fos-labeled neurons observed in the dorsal horns in both the RAO and RVO animals.

In summary, these data have provided a functional map of spinal pathways that are activated by renal receptors that respond to RVO or RAO and suggest that major classes of renal receptors have specific central pathways.

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