c-Fos generation in the dorsal vagal complex after systemic endotoxin is not dependent on the vagus nerve

G. E. HERMANN, G. S. EMCH, C. A. TOVAR, AND R. C. ROGERS
Department of Neuroscience, Ohio State University, Columbus, Ohio 43210

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Hermann, G. E., G. S. Emch, C. A. Tovar, and R. C. Rogers. c-Fos generation in the dorsal vagal complex after systemic endotoxin is not dependent on the vagus nerve. Am J Physiol Regulatory Integrative Comp Physiol 280: R289–R299, 2001.—The present study used activation of the c-Fos oncogene protein within neurons in the dorsal vagal complex (DVC) as a marker of neuronal excitation in response to systemic endotoxin challenge [i.e., lipopolysaccharide (LPS)]. Specifically, we investigated whether vagal connections with the brain stem are necessary for LPS cytokine-induced activation of DVC neurons. Systemic exposure to LPS elicited a significant activation of c-Fos in neurons in the nucleus of the solitary tract (NST) and area postrema of all thiobutabarbital-anesthetized rats examined, regardless of the integrity of their vagal nerves. That is, rats with both vagi cervically transected were still able to respond with c-Fos activation of neurons in the DVC. Unilateral cervical vagotomy produced a consistent but small reduction in c-Fos activation in the ipsilateral NST of all animals within this experimental group. Given that afferent input to the NST is exclusively excitatory, it is not surprising that unilateral elimination of all vagal afferents would diminish NST responsiveness (on the vagotomized side). These data lead us to conclude that the NST itself is a primary central nervous system detector of cytokines.

lipopolysaccharide; brain stem; nucleus of the solitary tract; vagotony

Cytokines are released by activated macrophages and lymphocytes as part of the immune response to antigenic challenge, injury, or irradiation. Elevation of the early proinflammatory cytokine tumor necrosis factor (TNF)-α in the systemic circulation has been correlated with anorexia, nausea, vomiting, and gastrointestinal stasis (7, 22, 23). This correlation between elevated plasma cytokine levels and changes in physiological state associated with illness implies a communication between the immune and nervous systems. Recent evidence suggests that the dorsal vagal complex (DVC) in the medulla oblongata may be one locus for TNF-α action to control gastrointestinal function (13, 19, 20).

The DVC consists of the sensory nuclei of the solitary tract (NST), the area postrema (AP), and the dorsal motor nucleus of the vagus (DMN). These nuclei comprise the final common pathway of the vago-vagal reflex circuits that control gastric motility (37). This medullary brain stem area has been identified as possessing the characteristics of a circumventricular organ and is essentially devoid of a blood-brain barrier (4, 18, 48). In addition, previous anatomic work (35, 36, 43) demonstrated that dendritic endings of neurons in both the NST and the DMN penetrate the AP and the floor of the fourth ventricle. Together, these anatomic characteristics place the DVC in a position to monitor blood-borne and cerebrospinal fluid-borne factors and to change vagally mediated autonomic functions accordingly (9, 27, 37). The brain stem has a high density of TNF-α binding sites (24) and is in a position to monitor blood-borne peptides. Therefore, it was hypothesized that the DVC may be a site at which circulating TNF-α acts to provoke gastric stasis and the other prodromata of illness such as nausea and emesis.

Endogenous production of TNF-α can be readily elicited by systemic administration of the bacterial cell coat component, lipopolysaccharide (LPS) (47). Our previous studies (20) demonstrated that TNF-α production in response to systemic (i.e., intravenous) LPS is sufficient to suppress centrally stimulated increases in gastric motility. Our earlier study demonstrated that TNF-α injected unilaterally into the DVC abolished a centrally stimulated and vagally dependent increase in gastric motility in a dose-dependent manner (19). The rapidity of the centrally injected TNF-α effect on gastric motility, i.e., within 30 s of application to the DVC, suggested that TNF-α could directly and rapidly affect the firing rate of neurons in the DVC. Electrophysiological studies by Emch et al. (13) showed that neurons of the NST that form the sensory limb of a vago-vagal gastroinhibitory reflex (35, 42) are strongly activated by doses of TNF-α previously shown to affect gastroinhibition (19).

Work by Sehic and Blatteis (3, 42) and others (14, 15, 17, 28) describes a potential alternate pathway by which information about immune activation may be transmitted to the central nervous system (CNS). There is evidence suggesting that vagal afferents, especially those in the hepatic branch, contain receptive elements responsive to cytokine or complement levels (3, 44). The mechanism implied is similar to that re
sponsible for the integrative physiological and behavioral actions of CCK. In this case, CCK, released by duodenal enterocytes, activates vagal afferent fibers that, in turn, produce a suppression of gastric motility as well as food intake (33, 41). The hypothesis had been made that systemic levels of cytokines are monitored by vagal afferents in the periphery, and their activation is responsible for illness behaviors and physiological responses such as fever and gastrointestinal malaise (14, 15, 17, 28). However, the role for vagal afferents in the transmission of information about peripheral cytokine release provoking gastrointestinal malaise and other illness behaviors has been called into question recently. That is, some investigators have shown that elimination of vagal afferent pathways does not block the malaise-inducing, anorexic, somnogenic, or febrile effects of cytokines (6, 21, 31, 41).

However, it is possible that both the vagal afferent and direct NST mechanisms operate parallel to monitor the portal circulation and the general systemic circulation, respectively. This hypothesis is supported by some reports that CNS effects (i.e., fever, illness behavior, etc.) of either low-dose intravenous or intraperitoneal LPS may be blunted by vagotomy, whereas the same CNS effects after high-dose intravenous LPS administration are not blocked by vagotomy (6, 21, 39, 42).

We decided to test the hypothesis that vagal pathways are essential to the CNS signaling of peripheral cytokine production by using the generation of the protein product of the proto-oncogene c-Fos as an anatomic identification of functionally activated neurons (32) in the NST. Previous studies (12, 45) showed that LPS-induced cytokine generation produces a significant increase in c-Fos labeling of neurons in the NST, i.e., the neurons in the medulla that receive vagal afferent projections. However, this earlier work did not establish whether the NST neurons were activated directly by circulating cytokine action or by vagal afferent pathways. It should be noted, however, that studies by Gaykema et al. (15) showed that subdiaphragmatic vagotomy abolished c-Fos expression in vagal sensory ganglia after intraperitoneal administration of LPS but only attenuated c-Fos expression in these nuclei when LPS was administered intravenously. These results suggest that different or redundant pathways are employed to inform the CNS about peripheral levels of cytokines.

The majority of the hepatic vagal afferents (i.e., the most likely peripheral afferent target for cytokines; Ref. 44), in addition to the usual complement of general visceral afferents from the thorax and abdomen, ascends via the left cervical vagal trunk (2, 34). Therefore, we propose the following hypotheses. 1) If intact vagal pathways are critical to the transmission of information to the NST concerning peripheral cytokine generation, then section of the left cervical vagal trunk should eliminate c-Fos generation in the NST on the side of section. 2) If direct action of cytokines at the NST is primary, then vagal transection should have no effect on NST c-Fos generation, and the numbers of c-Fos-labeled nuclei on both sides of the NST should be comparable. 3) If vagal afferent inputs modulate NST neurons that are, themselves, sensitive to levels of cytokines, then vagal section will reduce but not eliminate c-Fos generation by the NST.

These studies were designed to investigate three specific questions. First, it was necessary to establish that thiobutabarbital (Inactin) anesthesia would not interfere with c-Fos activation of NST neurons in response to endotoxin challenge and subsequent cytokine production. Furthermore, we wanted to determine if there was an intrinsic “sidedness” to the c-Fos distribution after peripheral LPS challenge. Given that the hepatic vagal afferents terminate principally in the left NST (34), if hepatic afferents constitute the primary pathway for the neurotransduction of peripheral cytokine level production (3, 14, 15, 17, 28, 42, 44), then we would expect more c-Fos activation on this side of the brain stem. The second set of experiments was designed to determine whether transection of the left cervical vagal trunk (with its predominance of hepatic afferent components) would eliminate c-Fos activation in the NST, indicating that vagal afferents were the critical conduits signaling the CNS of peripheral immune activation. The third set of experiments were performed on bilateral, cervical vagotomized rats to determine if loss of all vagal afferents (and efferents) would prevent c-Fos activation of NST neurons in response to endotoxin challenge via either intravenous or intraperitoneal routes.

METHODS

Drugs and Chemicals

Rats were anesthetized with thiobutabarbital (100 mg/ml; 100 mg/kg ip; Inactin; Research Biochemicals International, Natick, MA) dissolved in saline. This thiobutabarbital compound has been shown not to interfere with brain stem autonomic reflexes or with the generation of cytokines after the administration of LPS (5, 26). Endogenous production of TNF-α was induced by systemic administration of LPS. LPS was derived from Escherichia coli serotype 0111:B4 (Sigma; Ref. 47) and suspended in PBS (pH 7.4).

Histological processing of the medullary brain stem for c-Fos production required primary c-Fos antibody (Oncogene Science Diagnostics, Cambridge, MA; AB-5; rabbit c-Fos, 1:20,000) and biotinylated goat, anti-rabbit IgG (Vector Labs, Burlingame, CA; 1:600). Amplification of antibody-antigen reactions required incubation with Vector elite avidin-biotin-peroxidase complex (Vector Labs; 1:600) followed by Vector SG peroxidase detection reagents (Vector Labs).

Experimental Design

Experiment 1 was designed to establish that thiobutabarbital (Inactin) anesthesia would not interfere with c-Fos activation of NST neurons in response to endotoxin challenge and subsequent cytokine production. Second, we also wanted to determine if there was a dominant “side” effect of NST c-Fos production after systemic LPS exposure (see discussion above regarding asymmetric termination of hepatic afferents in the NST). Studies were performed in Inactin-anesthetized, vagally intact rats that were exposed to equivalent intrave-
nous volumes (0.1 ml/100 g body wt) of either PBS \((n = 4)\) or LPS \((1,000 \mu g/kg body wt; n = 6)\).

**Experiment 2** was designed to determine whether transection of the left cervical vagal trunk (with its predominance of hepatic afferent components) would eliminate c-Fos activation in the NST. These studies were performed in unilateral (i.e., left), cervical vagotomized rats that were exposed to one of four drug conditions: 1) PBS \((n = 4)\), 2) 1,000 \(\mu g/kg\) LPS dose \((n = 6)\), 3) 100 \(\mu g/kg\) LPS dose \((n = 6)\), or 4) 25 \(\mu g/kg\) LPS dose \((n = 6)\).

**Experiment 3** was designed to determine if loss of all vagal afferents (and efferents) would prevent c-Fos activation of NST neurons in response to endotoxin challenge via either intravenous or intraperitoneal routes. Thus these studies were performed in bilateral, cervical vagotomized rats. Given that other studies have suggested that lower doses of intravenous or intraperitoneal LPS may be more susceptible to vagotomy (6, 21, 39, 42), these studies only included our lowest dose of LPS. Bilateral vagotomized rats were exposed to one of three drug conditions: 1) intravenous PBS \((n = 4)\), 2) intravenous 25 \(\mu g/kg\) LPS \((n = 6)\), or 3) intraperitoneal 25 \(\mu g/kg\) LPS \((n = 5)\).

All three experiments used systemic administration of LPS to induce endogenous cytokine production. Our “high” dose of LPS \((1,000 \mu g/kg body wt)\) has been shown to be sufficient to induce substantial TNF-\(\alpha\) secretion (26) and to produce a significant gastric stasis under similar anesthetic conditions (20). This dose also produces a modest but consistent hypotension. Our “intermediate” dose \((100 \mu g/kg body wt)\) has been shown to elicit anorexic effects (31, 41) or fever (28) in awake rats. Finally, our “low” dose \((25 \mu g/kg)\) has been shown to effectively elevate plasma TNF-\(\alpha\) levels (16), produce fever, elicit c-Fos expression in the central amygdala, but does not provoke hypotension (45). The effects (or lack thereof) of LPS on blood pressure at these doses (i.e., 25, 100, or 1,000 \(\mu g/kg)\) were verified under Inactin anesthesia in our preliminary studies (data not shown).

Endogenous TNF-\(\alpha\) production reaches maximal plasma levels within 90 min of systemic administration of LPS (47). Studies by Rinaman et al. (32) demonstrated that maximal nuclear c-Fos immunoreactivity is present ~60–90 min following the presence of the presumptive stimulus. Therefore, comparable to other studies on c-Fos activation within the CNS after systemic exposure to endotoxin (40, 45), survival time before perfusion was selected to be 3 h after systemic injections of either PBS or LPS to maximize c-Fos activation expression. At the end of 3 h, rats were given a 0.2-ml bolus intravenous injection of lidocaine to stop respiration and cardiac function. The chest cavity was opened, and a blood sample by ventricular puncture was taken for subsequent ELISA verification of TNF-\(\alpha\) production. Animals were then transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. The brain stems were then removed to a solution of 4% paraformaldehyde and 20% sucrose in PBS to postfix for 16 h.

**Animals**

Male Long-Evans rats (Charles River Labs, Wilmington, MA) were maintained in a temperature-controlled vivarium with a 12:12-h day-night cycle. Animals had ad libitum access to food and water. All experimental procedures were performed according to guidelines set forth by the National Institutes of Health and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

### Surgical Preparations

Rats were anesthetized with Inactin \((100 \text{ mg/kg body wt ip})\). All subjects received tracheal cannulas to ensure the maintenance of an open airway for the duration of the experiment. Animals assigned to intravenous studies were equipped with sterile jugular cannulas. Depending on the assigned vagal status, each animal received one of three surgical manipulations: 1) exposure of cervical vagi without sectioning of the vagal trunks, i.e., intact \((n = 10)\), 2) left cervical vagal trunk section \((n = 22)\), or 3) bilateral cervical trunk section \((n = 15)\). In the rats receiving bilateral cervical vagotomies, the left vagus was cut ~20 min before the section of the right vagus. Although the rats developed apneic breathing after the section of the remaining vagus, all survived without auxiliary ventilation (38).

### Histological Processing for c-Fos Protein

Brain stems were sectioned on a freezing microtome at 50-\(\mu m\) thickness; sections were collected in PBS. After being rinsed in PBS, sections were treated with 1% sodium borohydride to reduce the fixative remaining in the tissue. After being rinsed in PBS, tissue sections were incubated for 1 h on a shaker in 10% normal sheep serum plus 0.3% Triton X in PBS to block nonspecific binding of the primary c-Fos antibody. After being rinsed, tissue sections were incubated in primary c-Fos antibody (Onco genie AB-5; rabbit c-Fos, 1:10,000) in 0.3% Triton in PBS for 16 h at room temperature with gentle agitation. Tissue sections were rinsed and incubated in biotinylated goat anti-rabbit IgG (Vector, 1:600) for 1 h. Sections were rinsed and reacted with Vector elite avidin-biotin-peroxidase complex (1:600 in PBS) for 1 h, followed by Vector SG peroxidase detection reagents. Specificity of the c-Fos immunocytochemical reaction was verified by omitting the c-Fos antibody from randomly selected sections. Sections were rinsed, mounted on glass slides, dried, cleared in Hemo-De (Fisher Scientific, Pittsburgh, PA), and placed under a coverslip with Entellan (Electron Microscopy Sciences, Fort Washington, PA).

### Counting c-Fos Nuclei in the Dorsal Medulla

c-Fos-labeled nuclei were counted manually with the aid of an MD2 Microscope Digitizer (Minnesota Datametrics, St. Paul, MN) encoder attached to the stage of a Leitz Dialux Microscope. Inclusion of c-Fos-labeled neurons required that the nuclei be a minimum of 6 \(\mu m\) in diameter and exhibit a nucleolus. These criteria guaranteed that staining artifacts and nuclear fragments would not be included in the count (see Figs. 2, 4, and 6). c-Fos-stained nuclei were counted without knowledge of the experimental condition, and counts were verified by a second observer. The agreement between counts of the two observers was within 5%. c-Fos activation of medullary neurons was analyzed at four specific coronal levels for each animal: 0.5 mm posterior to the calamus scriptor, the level of the calamus, the level of the area postrema (0.5 mm anterior to calamus), and the level of the anterior NST (1.0 mm anterior to calamus). The cumulated number of activated cells was totaled for the right and left side of each animal’s brain for statistical analysis. The distribution of labeled nuclei from the NST, AP, and DMN regions was analyzed separately.

### TNF-\(\alpha\) Assay

Plasma TNF-\(\alpha\) was determined by an ELISA kit for rat TNF-\(\alpha\) (R & D Systems, Minnesota, MN). Fifty-microliter plasma samples, in duplicate, were incubated at room tem-
temperature in microwells precoated with monoclonal anti-rat TNF-α antibody. After a 2-h incubation, each well was aspirated and washed with wash buffer; this process was repeated four times. One-hundred microliters of antibody against rat TNF-α conjugated to horseradish peroxidase was added to each well. After a 2-h incubation, each well was aspirated and washed with wash buffer; this process was repeated four times. One-hundred microliters of a tetramethylbenzidine peroxidase substrate was added to all wells. After a 30-min incubation at room temperature, the reaction was stopped by addition of hydrochloric acid. The optical absorbance of each well was read within 30 min with a microplate reader set to 450 nm. Absorbance values were converted to TNF-α concentrations by comparison with a simultaneously generated standard curve. The limits of detection per well of this assay kit were 12.5–800 pg/ml; the intersample and intra-assay variabilities were 8.8 and 2.1%, respectively (manufacturer’s data).

Analysis

Although animals were randomly assigned to one of the three surgical groups and experiments were run simultaneously, the analyses of c-Fos data were segregated according to surgical condition, i.e., 1) intact vagi, 2) left cervical vagotomy, and 3) bilateral cervical vagotomy, for two reasons. Most importantly, these three surgical manipulations result in rats with different physiological states (e.g., anesthetic vs. normal breathing) that may be reflected in basal (i.e., PBS challenge) conditions of c-Fos activation of DVC neurons. Second, these three experiments were designed to address different aspects of the DVC response to systemic endotoxin challenge.

Experiment 1: intact vagi/Inactin (thiobutabarbitral) anesthesia. Previous studies of CNS c-Fos production after LPS have been performed in unanesthetized rodents (12, 40, 45). Studies have shown that different anesthesia types may affect the immune response to LPS challenge, e.g., the reduction of TNF-α production under urethan anesthesia (20, 26). Therefore, it was necessary to first establish that vagally intact, Inactin-anesthetized rats were capable of inducing c-Fos synthesis in response to intravenous LPS challenge.

Second, recent data suggest that the significant cytokine-sensitive sensory pathway to the CNS arises from hepatic vagal afferents (44) that are represented asymptomatically within the two cervical vagal trunks and the NST. That is, the large majority of hepatic vagal afferents travels in the left cervical vagus and terminates in the left medial NST (2, 34). Therefore, the second aim of this experiment was to determine whether there was any intrinsic left versus right “sidedness” to the distribution of c-Fos in the brain stem of rats with both vagi intact.

The cumulated number of activated cells (i.e., total number of c-Fos-activated neurons from the 4 coronal sections) was totaled for the right and left side of each animal’s brain within either the NST or DMN. The NST and DMN c-Fos count results were independently subjected to Student’s t-tests.

AP c-Fos counts were obtained from the single sample section that contained this midline structure. Given that left versus right “sidedness” was not an issue with this structure, c-Fos-activated cell counts of PBS- versus LPS-challenged rats were analyzed using a Student’s t-test.

Experiment 2: left cervical vagotomy. Animals received left cervical vagotomy to eliminate vagal connections with half of the brain stem (i.e., the half that may receive a physiologically significant hepatic afferent projection). These unilaterally vagotomized rats were challenged with one of three doses of intravenous LPS or PBS. The cumulated number of activated cells (i.e., total number of c-Fos-activated neurons from the 4 coronal sections) was totaled for the right and left side of each animal’s brain within either the NST or DMN. The NST and DMN c-Fos count results were independently subjected to a repeated-measures ANOVA (i.e., left and right sides from the same animal; PBS vs. LPS groups; Ref. 29). In the event of a significant F value (i.e., F < 0.05), Dunnett’s posttests were used. Given that the AP is a midline structure (i.e., no right or left side), c-Fos-activated cell counts within the AP of the four conditions (i.e., PBS 25 μg/kg LPS, 100 μg/kg LPS, or 1,000 μg/kg LPS) were analyzed by one-way ANOVA.

Experiment 3: bilateral cervical vagotomy. It could be argued that any c-Fos label observed in the dorsal medulla ipsilateral to the unilateral vagotomy might be attributed to afferent activity from the remaining intact vagal trunk. Therefore, in this experiment, rats received bilateral cervical vagotomies to totally eliminate vagal connections with the NST. These animals received either intravenous PBS or our lowest LPS dose (25 μg/kg body wt) via either intravenous or intraperitoneal routes. As in the previous experiment, the cumulated number of activated cells (i.e., total number of c-Fos-activated neurons from the 4 coronal sections) was totaled for the right and left side of each animal’s brain.

Table 1. Summary of c-Fos-activated neurons

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<td></td>
<td></td>
<td>NST</td>
<td>DMN</td>
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<td></td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
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<td>PBS</td>
<td>64.5 ± 10.9</td>
<td>57.25 ± 13.8</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>15.3 ± 7.3</td>
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<td>1,000 μg/kg LPS</td>
<td>275.8 ± 13.3</td>
<td>272 ± 23.1</td>
<td>10.3 ± 2.2</td>
<td>10.5 ± 1.8</td>
<td>106.0 ± 18.1</td>
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<td>CVX</td>
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<tr>
<td>PBS</td>
<td>38.3 ± 12.0</td>
<td>54.0 ± 19.6</td>
<td>1.7 ± 0.6</td>
<td>1.9 ± 0.7</td>
<td>3.0 ± 1.9</td>
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<tr>
<td>25 μg/kg LPS</td>
<td>125.6 ± 5.4</td>
<td>148.7 ± 11.3</td>
<td>3.0 ± 0.6</td>
<td>3.7 ± 1.1</td>
<td>64.0 ± 10.0</td>
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<tr>
<td>100 μg/kg LPS</td>
<td>204.5 ± 29.6</td>
<td>263.0 ± 44.7</td>
<td>11.2 ± 4.4</td>
<td>12.2 ± 4.1</td>
<td>100.2 ± 26.4</td>
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<tr>
<td>1,000 μg/kg LPS</td>
<td>179.2 ± 33.6</td>
<td>263.7 ± 33.8</td>
<td>7.8 ± 2.9</td>
<td>8.5 ± 2.7</td>
<td>95.7 ± 22.1</td>
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<tr>
<td>BVX</td>
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<tr>
<td>PBS</td>
<td>49.3 ± 11.0</td>
<td>54.0 ± 11.5</td>
<td>5.5 ± 2.2</td>
<td>5.3 ± 2.6</td>
<td>12.0 ± 1.9</td>
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<td>25 μg/kg LPS (iv)</td>
<td>124.7 ± 13.7</td>
<td>126.5 ± 15.5</td>
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<td>25 μg/kg LPS (ip)</td>
<td>148.2 ± 10.1</td>
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<td>4.2 ± 1.1</td>
<td>5.8 ± 1.8</td>
<td>42.6 ± 6.7</td>
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Number of c-Fos-activated cells (means ± SE) in nucleus of solitary tract (NST), dorsal motor nucleus (DMN), and area postrema (AP) of Inactin-anesthetized, rats under 1 of 3 surgical conditions [i.e., intact vagi, unilateral cervical vagotomy (CVX), or bilateral cervical vagotomy (BVX)] in response to systemic challenges of PBS or lipopolysaccharide (LPS), as designated.
within either the NST or DMN. The NST and DMN c-Fos count results were independently subjected to a repeated-measures ANOVA (i.e., left and right sides from the same animal; PBS vs. LPS groups; Ref. 29). c-Fos-activated cell counts within the AP of the three different conditions [i.e., PBS, LPS (intravenous), or LPS (intraperitoneal)] rats were analyzed by one-way ANOVA.

Plasma TNF-α Levels

Plasma TNF-α levels were analyzed according to the systemic challenge (i.e., PBS or different doses of LPS; n = 35) by using the Kruskal-Wallis test for nonparametric samples. Statistical significance was defined as an overall P < 0.05; Dunn’s multiple-comparison posttests were applied.

RESULTS

Experiment 1: c-Fos Labeling in the NST, DMN, and AP in the Vagus-Intact, Inactin-Anesthetized Rat

Systemic LPS challenge induced a significant rise in NST c-Fos labeling in vagus-intact, Inactin-anesthetized rats (Table 1; Figs. 1A and 2; t = 11.3, df = 8, P < 0.0001). This elevation in NST c-Fos count after intravenous LPS was symmetrical in intact nonvagotomized rats; i.e., there was no intrinsic difference in distribution (sidedness) of NST neurons c-Fos activated in response to systemic endotoxin challenge (P = 0.3223). If hepatic afferents (which ascend predominantly within the left cervical vagal trunk to terminate within the left NST) were the principal pathway by which systemic exposure to endotoxin provoked c-Fos activation of brain stem neurons, then one might have expected more NST neurons to be labeled on the left as opposed to the right side.

Systemic LPS challenge produced a statistically significant but small increase in the numbers of DMN neurons containing c-Fos nuclear staining (Table 1; Figs. 1B and 2; t = 3.612, df = 8; P = 0.0069). Again, there was no difference in numbers of c-Fos-labeled nuclei between right and left sides of the brain stem.
c-Fos labeling of neurons in the AP was also significantly increased by LPS challenge in the vagus-intact rat (Table 1; Figs. 1C and 2; t = 4.658; df = 6; P = 0.0035).

Experiment 2: Effects of LPS on c-Fos Labeling in Rats with Left Cervical Vagotomy

Animals with left cervical vagotomy demonstrated a significant increase in c-Fos activation of NST neurons, regardless of dose of intravenous LPS or side of brain stem sampled (i.e., ipsilateral or contralateral to vagotomy) (Table 1; Figs. 3A and 4; F = 9.71; df = 3,17; P = 0.006; Dunnett’s posttest, P < 0.05).

The effect of unilateral vagotomy on the distribution pattern of c-Fos-activated neurons (i.e., left vs. right) was not significant at any individual LPS dose. However, when the data were collapsed across all groups, including the PBS controls, the right side (i.e., nonvagotomized) of each brain stem contained somewhat more c-Fos-activated NST cells than the corresponding (or paired) left side. This sidedness was statistically significant as a consequence of the consistency of this observation across all groups as opposed to the actual magnitude of the difference within any particular group (F = 40.909, df = 1,17, P = 0.0001).

Although the number of c-Fos-labeled cells in the DMN was increased with LPS challenge (Table 1, Figs. 3B and 4), because of the overall small number of DMN cells activated, this increase was not statistically significant (F = 2.206, df = 3,17, P = 0.1298).

There was a significant increase in the number of c-Fos-labeled neurons in the AP in response to all doses...
Experiment 3: Effects of LPS on c-Fos Labeling in Rats with Bilateral Cervical Vagotomy

Animals with bilateral cervical vagotomy (i.e., devoid of vagal connections with the CNS) still demonstrated a highly significant elevation in the numbers of c-Fos-labeled neurons in the NST. This response to our low dose (25 mg/kg body wt) of LPS was evident whether delivered via either the intravenous or intraperitoneal route (Table 1; Figs. 5C and 6; $F = 15.24$, df = 2,12, $P = 0.0005$; Dunnett’s post hoc $P < 0.05$).

The number of DMN neurons showing c-Fos activation after endotoxin challenge (via either intravenous or intraperitoneal routes) in these bilaterally vagotomized animals was comparable to the numbers seen in either the unilaterally vagotomized or vagally intact groups. However, this c-Fos response is not different from that elicited by PBS under these physiological conditions (Table 1; $F = 2.206$, df = 3,17, $P = 0.1298$).

Finally, the number of c-Fos-activated cells in the AP following endotoxin challenge was significantly increased in the bilateral, cervical vagotomized groups (Table 1; Figs. 5C and 6; $F = 5.108$, df = 2,10, $P = 0.0296$). Dunnett’s posttest revealed that only the LPS (intravenous) group was statistically significant relative to the PBS group.

**Plasma TNF-α Levels**

Blood samples were obtained at ~180 min post injection of either PBS or one of the different doses of LPS, i.e., immediately before transcardial perfusion for histological processing. All three doses of LPS (25, 100, or...
1,000 μg/kg body wt) elicited significant production of circulating TNF-α in Inactin-anesthetized rats regardless of route of administration (i.e., intravenous or intraperitoneal) or integrity of the vagal nerve trunks (i.e., intact, unilateral, or bilateral vagotomy) (Fig. 7; n = 35; Kruskal-Wallis test $P = 0.0001$; Dunn’s post-test $P < 0.05$).

Although there was no significant difference between the amounts of TNF-α elicited by the different doses of LPS across the various groups, there was a highly significant correlation (Spearman $r = 0.6908$; $P < 0.0001$) between levels of plasma TNF-α and number of c-Fos-activated neurons in the NST.

DISCUSSION

These studies demonstrated that, even in Inactin-anesthetized rats, neurons in the NST and AP demonstrate a significant increase in c-Fos nuclear protein labeling after systemic challenge of endotoxin (i.e., via either intravenous or intraperitoneal routes) regardless of the integrity of the vagus nerves (i.e., intact, unilateral, or bilateral cervical vagotomy). This increase occurs at both hypotensive and nonhypotensive doses of LPS, suggesting that the c-Fos labeling is a primary effect of LPS-induced cytokine production on the nervous system, as opposed to a primary effect on peripheral vasculature that is signaled by baroreceptive afferents (10).

Additionally, from the unilateral vagotomy experiments, our data indicate that the vagus nerve exerts a subtle effect on the responsiveness of the NST to other afferent inputs. That is, in the unilateral vagotomized groups (experiment 2), c-Fos labeling was modestly but consistently depressed on the vagotomized side of the brain stem (i.e., left side) at all doses of LPS as well as in the control, PBS, group. Only when the results from all doses were combined does the effect of vagotomy on the distribution of c-Fos-activated cells in the NST achieve statistical significance. Although one cannot rule out the possibility that cutting the vagus removes an important pathway regarding information concerning peripheral cytokine levels (3, 15, 44), it is more likely that this uniform reduction in c-Fos labeling in the NST is due to removal of a significant source of general vagal afferent excitation. That is, general visceral afferent input to the NST is glutaminergic and this input is responsible for a majority of the tonic excitation that NST neurons receive (37). Therefore, removal of vagal inputs to the NST, regardless of the afferent modality, will reduce the overall excitability of NST neurons (30). Nevertheless, the effects of systemic exposure to LPS were certainly detectable by neurons within the NST regardless of the connectivity of any reduction in sensitivity caused by the removal of vagal afferents. Second, the number of c-Fos-activated NST neurons was highly correlated with TNF-α production.

Our studies employed unilateral and bilateral cervical vagotomies (i.e., caudal to the nodose ganglia). Although nodose neurons may still detect LPS-related signals and communicate them to the CNS through their intact synaptic inputs to the DVC, studies by Gaykema et al. (15) have already shown that subdiaphragmatic vagotomy (i.e., even more caudal vagotomies) abolished c-Fos expression in vagal sensory ganglia after intraperitoneal administration of LPS and attenuated c-Fos expression in these nuclei when LPS was administered intravenously (15). Furthermore, elimination of vagal afferent pathways rostral to the
nodose (31, 41) does not block the anorexia produced by peripheral LPS. Taken together, these results suggest that different or redundant pathways (i.e., both neural and humoral) are employed to inform the NST about circulating levels of cytokines.

c-Fos labeling of cells in the AP parallels that of the NST. It is possible that the AP is the principal CNS detector of cytokines elicited by the LPS challenge. That is, NST labeling may only be a consequence of excitatory inputs from the AP (46). However, morphological and physiological studies do not support the concept of the AP acting only as a specialized chemosensor, with the NST acting only as a processor of general visceral afferent information from the vagus. Rather, the NST and AP share several morphological and functional features: 1) both nuclei receive primary vagal afferent inputs (34), 2) both nuclei are vascularized by fenestrated capillaries (18), and 3) dendrites from the NST (and DMN) are intermingled within the AP (43). It is likely that the c-Fos-labeled NST and AP neurons share a common sensitivity to cytokines and that AP neurons modulate NST and DMN excitability (46).

In vagally intact animals, systemic challenge with endotoxin also resulted in a statistically significant increase in c-Fos labeling in both the NST and AP compared to PBS controls. Post hoc testing indicated that only the LPS (intravenous) group was statistically significant relative to the PBS group (F = 5.108; df = 2,10; P = 0.0296; Dunnett’s posttest *P < 0.05).

Fig. 5. Experiment 3: graphic representation of cell counts (means ± SE) of c-Fos-activated neurons in the brain stem of bilateral, cervical vagotomized rats that received PBS or LPS (intravenous or intraperitoneal) while under Inactin anesthesia. A: animals with bilateral cervical vagotomy demonstrated a significant increase in c-Fos-labeled neurons in the NST in response to 25 μg/kg LPS, regardless of the route of administration (F = 15.24, df = 2,12, P = 0.0005; Dunnett’s posttest *P < 0.05). B: systemic LPS exposure did not significantly increase the number of c-Fos-positive neurons in the DMN above levels seen after PBS injections (P = 0.7876). C: LPS challenges (both intravenous and intraperitoneal routes) significantly increased c-Fos labeling in the AP. Post hoc testing indicated that only the LPS (intravenous) group was statistically significant relative to the PBS group (F = 5.108; df = 2,10; P = 0.0296; Dunnett’s posttest *P < 0.05).

Fig. 6. Micrographs of c-Fos production in the DVC of rats with bilateral cervical vagotomy. Bilaterally vagotomized rats are still capable of responding with an increase in c-Fos production in neurons of the DVC after systemic (either intravenous or intraperitoneal) challenge with endotoxin. A: c-Fos production in response to intravenous PBS; B: 25 μg/kg LPS (intravenous); C: 25 μg/kg LPS (intraperitoneal). Scale bar = 0.5 mm.
incurred to the DVC area containing these NST neurons also shown that subfemtomole doses of TNF-α. Parallel studies (19) have the early cytokine TNF-(36, 49) are directly activated by subfemtomole doses of produced. Furthermore, neurophysiological studies in our laboratory (13) show that NST neurons responsible for coordinating reflex inhibition of gastric function for the vagal afferent and descending CNS afferent influences (37, 48). The NST is in position to directly transduce this “hormonal” signal into changes in excitability, although it is extremely likely that NST activity (perhaps, also the DMN) is indirectly modulated by chemosensor neurons in the AP, which, in turn, synapse on NST or DMN neurons (46).

Perspectives

These results provide evidence that the NST and AP response to the effects of LPS challenge is mediated locally and does not require the presence of intact vagal afferent innervation. This finding is consistent with previous results showing that these structures directly sense humoral “afferent” signals and control important autonomic and behavioral functions accordingly (27). That is not to say that vagal afferent mechanisms that detect the consequences of immune activation do not exist, but rather that other pathways (i.e., humoral) exist that are sufficient to provide direct CNS activation after immune challenges. Note that these results do not confound previous observations of a primary vagal afferent mechanism that detects the consequences of immune activation that may result in a febrile response (3, 44). It is likely that vagal afferents in the hepatic branch of the vagus are involved in detecting activation of the mucosal immune system of the gut. Cytokines and other markers of immune activation produced in the gastrointestinal tract are released into the portal circulation first and could be detected first by hepatic vagal afferents (3, 44). However, the physiological impact of this vagal afferent path must be very brief, because total cervical vagotomy did not block c-Fos production within the NST after intraperitoneal administration of endotoxin (i.e., a model for gastrointestinal immune activation).

It is very likely that the NST and vagal afferent cytokine detection pathways are further examples of serial detection mechanisms that the brain uses to regulate a variety of homeostatic processes. For example, neurons in the NST and general visceral afferent sensors (including the vagus) are sensitive to peptide hormones, CO₂ tension, glucose, sodium, osmolality, and temperature (Ref. 27, 11, 1, 25, and 8, respectively). Removal of the vagal afferent input to the NST may blunt, but not eliminate, the brain’s capacity to regu-
late physiological processes that control these parameters.

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