Effects of vagotomy on serum endotoxin, cytokines, and corticosterone after intraperitoneal lipopolysaccharide

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Hansen, Michael K., Kien T. Nguyen, Monika Fleshner, Lisa E. Goehler, Ron P. A. Gaykema, Steven F. Maier, and Linda R. Watkins. Effects of vagotomy on serum endotoxin, cytokines, and corticosterone after intraperitoneal lipopolysaccharide. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R331–R336, 2000.—The vagus nerve appears to play a role in communicating cytokine signals to the central nervous system, but the exact extent of its involvement in cytokine-to-brain communication remains controversial. Recently, subdiaphragmatic vagotomy was shown to increase bacterial translocation across the gut barrier and thus may cause endotoxin tolerance. The current experiment tested whether or not vagotomized animals have similar systemic responses to endotoxin challenge as do sham-operated animals. Subdiaphragmatically vagotomized and sham-operated animals were injected intraperitoneally with one of three doses (10, 50, 100 µg/kg) of lipopolysaccharide (LPS) or vehicle, and blood samples were taken at 15, 30, 60, 90, and 120 min after the injection. The intraperitoneal injection of LPS increased circulating LPS levels at all time points examined. In addition, all three doses of LPS significantly increased serum interleukin (IL)-1β, IL-6, and corticosterone in both control and vagotomized rats. In conclusion, vagotomy itself has no marked effect on circulating endotoxin levels or the production of IL-1β, IL-6, or corticosterone in blood after an intraperitoneal injection of LPS.

interleukin-1β; interleukin-6; vagus nerve; cytokine-to-brain communication

THERE HAS BEEN CONSIDERABLE recent interest in the mechanisms involved in immune-to-brain communication. It is generally thought that in response to an infection, cytokines are released and are important mediators in signaling the central nervous system (CNS) to elicit “sickness behavior,” which includes fever, increases in sleep, depression in food-motivated behavior, exaggerated pain responses, and activation of the hypothalamic-pituitary-adrenal (HPA) axis (19, 20, 32). Although there is considerable evidence for the existence of a number of mechanisms by which blood-borne cytokines, such as interleukin (IL)-1β, can signal the CNS (2, 29, 33), more recent studies have implicated a neural afferent pathway as well. Indeed, systemic IL-1β increases electrical activity of vagal afferents (8, 25), induces c-Fos in vagal primary afferent neurons (8, 14), and IL-1β binding and immunoreactivity have been localized to abdominal vagal afferents and paraganglia (13, 15). Furthermore, subdiaphragmatic vagotomy has blocked or attenuated a variety of the brain-mediated responses to peripheral immune challenge (3, 6, 22).

It is generally assumed that vagotomy blocks brain activation by interrupting afferent signaling. However, this assumption has recently come into question (26, 30) due to data suggesting increased bacterial translocation across the gut barrier in subdiaphragmatically vagotomized rats (7). Thus an alternative explanation for vagotomy blockade of brain activation and sickness behavior following the peripheral administration of agents such as lipopolysaccharide (LPS) is that slowed gastrointestinal transit after vagotomy may cause increased bacterial translocation across the gut and lead to endotoxin tolerance (e.g., reduced cytokine production by immune cells in the periphery in response to immune challenge) (30). In addition, it is plausible that vagotomy may alter the tendency of intraperitoneally injected LPS and/or cytokines to gain access to the general circulation. Thus the aim of the current experiment was to examine the distribution of endotoxin in the circulation after an intraperitoneal injection of LPS, and the levels of IL-1β, IL-6, and corticosterone in serum from sham-operated and subdiaphragmatically vagotomized rats.

METHODS

Animals. Adult male Sprague-Dawley rats (250 g at purchase; Harlan Sprague Dawley, Indianapolis, IN) were used in all studies. Animals (n = 64) were individually housed in hanging metal cages at 25 ± 1°C with a 12:12-h light-dark cycle (lights on at 0600). Standard rat chow and water were freely available unless otherwise noted. Care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Surgery. Subdiaphragmatically vagotomized (Vag) rats or sham-operated (Sham) rats were prepared under Halothane anesthesia as previously described in detail (34). During the immediate postsurgical period (~2 days), both Sham and Vag rats were maintained on highly palatable food and received acetaminophen (0.5 mg/ml) in their drinking water. Rats who did not gain weight were killed (2 out of 32 Vag rats).

Vagotomy assessment. Approximately 3 wk after surgery, the completeness of vagotomy was assessed using the food...
intake analysis test as previously described (16, 17). This test is based on the satiety effect of cholecystokinin (CCK), which is known to be mediated by the vagus nerve (31). In brief, on separate days, each rat was injected intraperitoneally with saline or 4 µg/kg CCK (CCK-octapeptide; Sigma, St. Louis, MO) after 20 h of food deprivation; a minimum of 3 days was allowed between the saline and CCK injections. Food intake was measured after 1 h in both Sham and Vag rats.

Experimental protocol. Experiments began ~1 wk after the completion of food intake analysis; thus ~5 wk after surgery. At the time of experimental testing, all animals were food deprived overnight but allowed access to water ad libitum in an attempt to normalize interindividual variation in gastrointestinal status (8). Sham (n = 32) and Vag (n = 30) rats received intraperitoneal injections of vehicle (sterile, pyrogen-free saline) or one of three doses (10, 50, or 100 µg/kg) of LPS (Escherichia coli, 0111:B4; Sigma, St. Louis, MO) 2 h after light onset in an injection volume of 1 ml/kg. Blood samples were taken from the tail vein at 15, 30, 60, and 90 min after the injection. The tail was cleaned with betadine prior to being nicked with a sterile #15 scalpel blade, and all blood samples were collected in sterile 1.5 ml microcentrifuge tubes. Rats were killed by decapitation 2 h after the injection, and trunk blood and peritoneal lavage fluid were collected. To collect peritoneal lavage fluid, 2 ml of sterile phosphate-buffered saline was added to the peritoneal cavity, after which the abdomen was gently massaged. Peritoneal lavage fluid (~1 ml) was collected in sterile 1.5 ml microcentrifuge tubes, centrifuged to remove cells (10,000 rpm, 10 min, 4°C), and stored at −20°C until assayed. Serum was separated by centrifugation (3,000 rpm, 20 min, 4°C) and stored at −80°C until time of assay. The liver was also dissected, snap-frozen in liquid nitrogen, and stored at −80°C until processed as described previously (24). Briefly, ~100 mg of liver tissue were sonicated in 1 ml of a sonication buffer containing 5% fetal calf serum, and an enzyme inhibitor cocktail consisting of 100 mM amino-N-capric acid, 10 mM EDTA, 5 mM benzamidine-HCl, and 0.2 mM phenylmethylsulfonyl fluoride (all from Sigma, St. Louis, MO). Sonicated samples were centrifuged (10,000 rpm, 10 min, 4°C) and supernatants were removed and stored at 4°C until assayed. In addition, Bradford protein assays were performed to determine total protein concentrations (5).

Assays. Endotoxin levels were measured using a chromogenic Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD) according to the manufacturer’s instructions. Before testing, serum was diluted 1:5 or 1:100, and peritoneal lavage fluid was diluted 1:5 or 1:1,000 with endotoxin-free water. IL-1β protein levels were measured using a commercially available ELISA kit (R & D systems, Minneapolis, MN). The validation and use of the kit has been previously described in detail (24). IL-6 was measured using a commercially available ELISA kit (BioSource International, Camarillo, CA) according to the manufacturer’s instructions with slight modifications. For the IL-6 ELISA, samples were diluted 1:4 before assay. Serum corticosterone was measured using a radioimmunoassay (ICN Pharmaceuticals, Costa Mesa, CA) according to the instructions provided.

Data analysis. The effects of vagotomy and LPS on serum IL-1β, IL-6, endotoxin, and corticosterone were analyzed across time using a three-way repeated measures ANOVA. If ANOVA indicated a significant surgery × drug × time interaction, a separate two-way ANOVA was performed on individual times to see where the significant differences occurred. IL-1β protein levels in the liver and endotoxin levels in the peritoneal cavity were evaluated by two-way ANOVA. When appropriate, post hoc analysis was done using the Student-Newman-Keuls multiple comparison test. In all tests, an α-level of P < 0.05 was taken as an indication of statistical significance. Three animals (1 Sham, 2 Vag) were excluded from analysis because we were unable to detect any LPS from peritoneal lavage fluid or serum in these animals, which received an intraperitoneal injection of LPS. The absence of LPS from the peritoneal cavity suggests that these rats may have received an improper injection.

RESULTS

Food intake. In this experiment, food intake analysis was used to assess the completeness of vagotomy. CCK significantly inhibited food intake in Sham (P < 0.05), but not in Vag rats. Food intake was decreased by 47% in CCK-injected Sham rats compared with the saline injection (3.46 ± 0.15 vs. 6.52 ± 0.19 g, respectively). In contrast, CCK did not significantly decrease food intake in Vag rats compared with the saline injection (5.42 ± 0.23 vs. 5.42 ± 0.24 g, respectively).

Ten microgram per kilogram LPS. The intraperitoneal injection of 10 µg/kg LPS increased circulating levels of LPS (Fig. 1A), resulting in a main effect of drug [F(1,25) = 36.429, P = 0.0001], whereas there was no main effect of surgery or surgery × drug × time interaction. LPS levels were significantly increased at all time points examined in both Sham and Vag animals, and there were no significant differences between the two groups. This dose of LPS also resulted in main effects of drug on serum IL-1β, serum IL-6, and serum corticosterone [F(1,25) = 71.302, 49.123, and 47.157, respectively; P < 0.0001 for each comparison]. Significant increases in serum IL-1, serum IL-6, and serum corticosterone were found at 60, 90, and 120 min after

Fig. 1. Effects of intraperitoneal lipopolysaccharide (LPS) or vehicle (saline) on circulating levels of endotoxin in subdiaphragmatically vagotomized (Vag) and sham-operated (Sham) rats. Results for Vag saline group are shown, but obscured by Sham saline group. Values are means ± SE. A: 10 µg/kg LPS; B: 50 µg/kg LPS; C: 100 µg/kg LPS.
the injection compared with the saline injection (Figs. 2, A and D, and 3A). There were no significant differences in the magnitude of the increases between Sham and Vag rats.

Fifty micrograms per kilogram LPS. The intraperitoneal injection of 50 µg/kg LPS increased circulating levels of LPS (Fig. 1B), resulting in a main effect of drug \[F(1,26) = 232.505, P < 0.0001\], whereas there was no main effect of surgery or surgery \(\times\) drug \(\times\) time interaction. LPS levels were significantly increased at all time points examined in both Sham and Vag animals, and there were no significant differences between the two groups. This dose of LPS also resulted in main effects of drug on serum IL-1β, serum IL-6, and serum corticosterone \[F(1,26) = 115.309, 229.178, \text{and} 82.121, \text{respectively}; P < 0.0001 \text{for each comparison}\]. Again, significant increases in serum IL-1, serum IL-6, and serum corticosterone were found at 60, 90, and 120 min after the injection compared with the saline injection (Figs. 2, B and E, and 3B). There were no significant differences in the magnitude of the increases between Sham and Vag rats.

One hundred microgram per kilogram LPS. This dose of LPS resulted in similar effects on circulating LPS levels and serum cytokines and corticosterone, as did the two lower doses (Figs. 1C, 2, C and F, and 3C). That is, the intraperitoneal injection of 100 µg/kg LPS increased circulating LPS levels, serum IL-1β, serum IL-6, and serum corticosterone compared with the saline injection in both Sham and Vag animals. However, there was a reduction in circulating levels of LPS in Vag rats compared with Sham rats, resulting in a

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**Fig. 2. Effects of intraperitoneal LPS or vehicle (saline) on serum interleukin (IL)-1β and IL-6 in Vag and Sham rats.** Results for Vag saline group are shown but obscured by Sham saline group. Values are means ± SE. A, D: 10 µg/kg LPS; B, E: 50 µg/kg LPS; C, F: 100 µg/kg LPS.

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**Fig. 3. Effects of intraperitoneal LPS or vehicle (saline) on serum corticosterone in Vag and Sham rats.** Values are means ± SE. A: 10 µg/kg LPS; B: 50 µg/kg LPS; C: 100 µg/kg LPS.
significant surgery × drug × time interaction [F(4,104) = 6.935, P < 0.0001]. Significant differences were found at the 15-, 30-, 60-, and 120-min time points (P < 0.05). In addition, the magnitude of the LPS-induced increases in serum IL-1β and IL-6 levels in the Vag animals compared with the Sham animals was reduced, resulting in significant surgery × drug × time interactions [F(4,104) = 6.62, P < 0.0001, and F(4,104) = 6.685, P < 0.0001; for IL-1β and IL-6, respectively]. This difference was due to significantly lower levels of IL-1β and IL-6 at the 90- and 120-min time points (P < 0.05). This dose of LPS again elevated serum corticosterone, resulting in a significant drug effect [F(1,26) = 106.604, P < 0.0001]. There was no significant difference in the magnitude of the increase between the LPS-injected Sham and Vag animals.

LPS in peritoneal lavage fluid and liver IL-1β. Intraperitoneal injections of LPS dose dependently increased LPS levels in the peritoneal lavage fluid [F(3,51) = 91.195, P < 0.0001; Fig. 4A]. Furthermore, at the time of death (2 h), LPS significantly increased IL-1β protein levels in the liver [F(3,51) = 114.553, P < 0.0001; Fig. 4B]. There were no significant differences in endotoxin levels in peritoneal lavage fluid or IL-1β levels in liver between Sham and Vag rats.

DISCUSSION

In control rats, all three doses of LPS increased circulating LPS levels at the earliest time point examined (15 min) and circulating levels remained elevated for the duration of the experiment (2 h). These data indicate that LPS quickly gains access to the general circulation but is not rapidly cleared from the circulation despite the clearing ability of the liver Kupffer cells (11). Furthermore, LPS dose dependently increased circulating IL-1β and IL-6, increased corticosterone beginning at 60 min after the injection, and increased liver IL-1β levels 2 h after the injection. These data are in agreement with the concept that cytokines are critical mediators in signaling the central nervous system to orchestrate the cascade of endocrine, autonomic, and behavioral processes collectively termed the acute phase response.

In addition, the current results do not support the hypothesis that vagotomy inhibits brain-mediated illness responses because it inhibits peripheral cytokine production (30). In contrast, with the two lower doses (10 and 50 µg/kg) of intraperitoneal LPS, no significant differences were found in circulating levels of LPS or in the production of serum IL-1β, IL-6, or corticosterone between Sham and Vag animals. Thus it does not appear that vagotomized rats are endotoxin tolerant. However, it is important to note that the vagotomy-associated bacterial translocation previously reported was only examined 7 days after surgery (7). The current experiments began ~5 wk after surgery, which is a similar recovery period used in prior vagotomy studies that found a blockade of various sickness behaviors by vagotomy (17, 28). Furthermore, there were no significant differences in circulating LPS levels in saline-injected Sham and Vag rats, which indirectly suggests that there is not an increase in bacterial translocation at this time. Nevertheless, it is not known whether vagotomy would alter cytokine production 1 wk after surgery when the increased bacterial translocation was reported to occur (7).

The 100-µg/kg dose of LPS resulted in many similar effects between Sham and Vag rats. However, there was a reduction in LPS-induced serum IL-1β and IL-6 at the 90- and 120-min time points. These differences in serum cytokines at the highest dose of LPS were not likely the result of endotoxin tolerance; rather, the decreases in serum IL-1β and IL-6 in Vag rats were likely due to the significant reduction in the amounts of LPS in the circulation at the earlier time points (e.g., 15 min; Fig. 1C). It remains unknown as to why differences in the transport of LPS from the peritoneal cavity occurred in this group, whereas no differences in transport were seen with the two lower doses of LPS. One possible explanation of these results is that the Vag rats in the highest-dose group (100 µg/kg) were in relatively “poor shape” compared with Vag rats in the lower-dose LPS groups. However, analysis of individual food intake and body mass data between the different groups of Vag rats did not reveal any differences between the groups, which allows us to exclude the...
possibility that the current results were due to poor health of the Vag rats in the highest-dose group or incomplete vagotomy in the two lower-dose groups. In addition, these differences at 100 μg/kg may be uncharacteristic, because in a similar study using 100 μg/kg LPS intraperitoneally, we did not find any significant differences in circulating levels of LPS or IL-1β between Sham and Vag rats (unpublished data). Furthermore, although differences in circulating IL-1β and IL-6 were found with the 100-μg/kg dose of LPS, there were no differences in serum corticosterone between Sham and Vag rats, suggesting that the differences in serum cytokines were not likely physiologically relevant differences. Also, there were no differences in liver IL-1β levels after any dose of LPS between Sham and Vag rats, suggesting that immune cells of liver, the Kupffer cells, are not tonically suppressed. Regardless of these considerations, it remains unknown whether the reduction in circulating cytokines observed in the current study at the 100-μg/kg dose of LPS would lead to differences in other brain activation measures or sickness behaviors commonly examined. Thus it is advisable to determine unequivocally whether circulating levels of LPS and/or cytokines are elevated equally in this type of experiment.

The current data also support earlier studies suggesting that vagotomy does not block or reduce cytokine-to-brain communication by means other than afferent interruption. Subdiaphragmatic vagotomy inhibits brain production of IL-1β mRNA in response to intraperitoneal IL-1β, yet does not alter liver IL-1β mRNA production (17). Vagotomy also does not alter plasma IL-1β levels or pituitary IL-1β mRNA, yet blocks IL-1β mRNA in the brain of mice in response to intraperitoneal LPS (21). In addition, vagotomy does not block fever in response to an intracerebroventricular injection of PGE2 (23) or behavioral effects of intracerebroventricular IL-1β (4), suggesting that vagotomy does not disrupt sickness responses by interrupting effector pathways or by impairing the direct sensitivity of the brain to immune signals. Finally, fever in response to intraperitoneal LPS is blocked by vagotomy in well-nourished rats, rejecting the possibility that the vagotomy-induced febrile nonresponsiveness is due to malnutrition (27). Collectively, these data support a direct action of vagal afferents in cytokine-to-brain communication and argue against several of the alternative hypotheses as to why vagotomy is blocking centrally controlled illness responses.

The lack of effect of vagotomy on circulating corticosterone requires comment, because vagotomy does blunt HPA activation to a variety of intraperitoneally administered immune-activating agents (9, 10, 12, 18). Vagotomy reduced corticosterone levels in response to intraperitoneal IL-1β and tumor necrosis factor-α (9, 10). In these studies, however, the blockade of corticosterone was only partial. In addition, in response to intraperitoneal LPS or IL-1β, vagotomy blocked adrenocorticotropic hormone secretion, yet had no effect on circulating corticosterone (12, 18). Finally, it is possible that direct action of IL-1β on the adrenal gland may contribute to corticosterone production (1), thus making the effects of vagotomy on corticosterone variable and difficult to interpret.

In conclusion, the suppressive effects of subdiaphragmatic vagotomy on various aspects of the acute phase response are not likely due to differences in the distribution of endotoxin in the circulation or in the peripheral production of cytokines. Rather, these data support the hypothesis that vagal afferent signaling is likely one mechanism by which cytokines can signal the brain to regulate centrally controlled aspects of the acute phase response.

Perspectives

The role of vagal afferents in cytokine-to-brain communication remains a topic of much debate. It is clear, however, that peripheral cytokines do signal the brain, and it is likely that this occurs through multiple routes. Alternative pathways include other vagal afferents that are still intact, other peripheral nerves, as well as various other routes of communication between the blood and brain, such as active transport mechanisms, passage at sites that lack a true blood-brain barrier (e.g., circumventricular organs), and barrier-cell mediated pathways (2, 29, 33). It is likely that peripheral cytokines use different routes of communication under specific circumstances. For example, the majority of the data suggests that vagal afferents likely contribute to cytokine-to-brain communication during relatively small challenges in the physiological range (16, 28), whereas other routes of communication may be more important during times of pathology when increases in cytokines gain access to sites closely linked to brain sites.

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VAGOTOMY DOES NOT PRODUCE ENDOTOXIN TOLERANCE


