LPS-induced suppression of gastric motility relieved by TNFR:Fc construct in dorsal vagal complex

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Hermann, Gerlinda E., C. Amy Tovar, and Richard C. Rogers. LPS-induced suppression of gastric motility relieved by TNFR:Fc construct in dorsal vagal complex. Am J Physiol Gastrointest Liver Physiol 284: G634–G639, 2003. First published April 24, 2002; 10.1152/ajpgi.00412.2001.—Our previous studies suggested that the cytokine tumor necrosis factor– (TNF–) may act within the neural circuitry of the medullary dorsal vagal complex (DVC) to affect changes in gastric function, such as gastric stasis, loss of appetite, nausea, and vomiting. The definitive demonstration that endogenously generated TNF–α is capable of affecting gastric function via the DVC circuitry has been impeded by the lack of an antagonist for TNF–α. The present studies used localized central nervous system applications of the TNF-adsorbant construct (TNFR:Fc; TNF-receptor linked to the Fc portion of the human immunoglobulin IgG1) to attempt to neutralize the suppressive effects of endogenously produced TNF–α. Gastric motility of thiobutabarbital-anesthetized rats was monitored after systemic administration of lipopolysaccharide (LPS) to induce TNF–α production. Continuous perfusion of the floor of the fourth ventricle with TNFR:Fc reversed the potent gastrointestinal induction by LPS, i.e., central thyrotropin-releasing hormone-induced increases in motility were not inhibited. This disinhibition of gastric stasis was not seen after intraventricular application of similar doses of TNF:Fc nor ventricular application of the Fc fragment of human immunoglobulin. These results validate our previous studies that suggest that circulating TNF–α may act directly within the DVC to affect gastric function in a variety of pathophysiological states. 

ACTIVATION OF A SYSTEMIC IMMUNE response by injury, infection, radiation, or chemotherapy is often accompanied by gastric stasis (which is perceived as nausea), loss of appetite, and vomiting (7, 32, 37). These pathophysiological symptoms are correlated with high circulating levels of proinflammatory cytokines, especially tumor necrosis factor–α (TNF–α) (10, 24, 32, 37). Indeed, systemic injections of TNF–α into human volunteers can mimic these characteristics of illness (20). Whereas it has been recognized that TNF–α plays a role in the anorexia associated with these different conditions, it is not clear whether these gastric effects are: 1) due to the direct effects of TNF–α or other cytokines triggered in the cytokine cascade or 2) mediated via central or peripheral sites of action of TNF–α.

The medullary dorsal vagal complex (DVC; composed of the sensory nuclei of the solitary tract (NST), the area postrema (AP), and the dorsal motor nucleus (DMN) of the vagus nerve) is the locus of the vagovagal reflex circuits that provide overall control of gastric motility (28). This brain stem area is considered to be a circumventricular organ in that it is essentially devoid of a blood-brain barrier (1, 12, 36). Additionally, dendritic endings of neurons in both the NST and the DMN have been reported to penetrate the AP and the floor of the fourth ventricle (26, 27, 30). Thus the DVC is in a position to monitor blood-borne and cerebrospinal fluid-borne factors and to change vagally mediated autonomic functions accordingly (5, 19, 28).

The brain stem has a high density of TNF–α binding sites (17, 23). Therefore, we have 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. Indeed, our earlier studies have already shown that nanoinjection of TNF–α directly into the DVC can: 1) completely suppress centrally mediated increases in gastric motility (14) even at doses of <1 fmol; 2) provoke fos activation of neurons in the NST (8); and 3) activate NST neurons belonging to gastric vagovagal reflex control circuits (7). This neurophysiological study also revealed that, once these gastric NST neurons were exposed (and responded) to TNF–α, subsequent responses of these neurons to otherwise innocuous visceral stimuli were highly potentiated. This finding coincides with the observation that cytokines, or agents that provoke cytokine production, evoke persistent, conditioned visceral aversion behavior (16). Perhaps the potentiating effect of TNF–α on NST neuron responses to visceral afferent input is critical to the production of such long-term changes in the responsiveness to visceral afferent input as learned taste aversion.

We have also shown that these same motility (15) and Fos-activation results (13) occur after endogenous production of TNF–α in response to systemic adminis-
tration of the endotoxin lipopolysaccharide (LPS). It is of interest to note that Fos-activation of NST and AP neurons occurred even when both cervical vagal trunks were transected, indicating that endogenous TNF-α is capable of accessing and activating neurons in the DVC directly (13). Together, these studies strongly suggest that one of the sites of TNF-α action is directly within the DVC to affect vagal sensory activity and, ultimately, gastric motility. Although compelling, direct proof would be more convincing. At present, no TNF-α antagonists are available. However, TNF-α-adsorbing constructs (e.g., TNFR:Fc; Enbrel) have been used clinically to bind and inactivate the proinflammatory effects of TNF-α in rheumatoid arthritis (21). Therefore, we hypothesized that if TNF-α is the active agent within the DVC to produce the above-cited changes in gastric motility, then local application (i.e., intracerebroventricularly) of TNFR:Fc should specifically block the effects of TNF-α endogenously generated after systemic administration of LPS. That is, endogenously produced TNF-α should be specifically inactivated by the TNFR:Fc construct in the DVC and centrally mediated activation of gastric motility should, again, be demonstrable.

METHODS AND MATERIALS

Drugs and chemicals. Rats were anesthetized with thiobutabarbital (100 mg/ml, 200 mg/kg ip; Inactin, Sigma, St. Louis, MO) dissolved in saline. This thiobutabarbital compound has been shown not to interfere with brain stem autonomic reflexes (2), the generation of cytokines after the administration of LPS (18), or the activation of DVC neurons after exposure to cytokines (7, 8, 14). Endogenous production of TNF-α was induced by systemic administration of LPS. LPS was derived from Escherichia coli serotype 0111:B4 (Sigma; Ref. 35) and suspended in PBS (124 mM NaCl, 26 mM NaHCO3, 2 mM KH2PO4, 304 mosmol/kgH2O, pH 7.4). Gastric motility can be stimulated centrally by exposing the floor of the fourth ventricle to 2 μl of a 100-μM solution of thyrotropin releasing hormone (TRH; Bachem, Torrance, CA) dissolved in PBS (14).

At the present time, no satisfactory antagonist for TNF-α receptors is available. However, physiological actions of TNF-α can be interrupted by synthetic TNF-receptor construct (Enbrel; Immunex, Seattle, WA), which acts to adsorb circulating TNF-α. TNF-receptor construct is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human p75 tumor necrosis factor receptor (TNFR) linked to the Fc portion of the human immunoglobulin, IgG1 (21). Each TNFR:Fc fragment molecule eliminates TNF-α by binding two TNF-α molecules, irreversibly. Application of the human IgG1 Fc fragment (Chemicon, Temecula, CA) was used as a control agent for the TNFR:Fc construct.

Animals. Male Long-Evans rats (200–400 g body wt; Simonsen Laboratories) were maintained in a temperature-controlled vivarium with a 12:12-h light-dark 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 preparation. Rats (n = 26) were anesthetized and received tracheal cannulae to ensure the maintenance of an open airway for the duration of the experiment. Animals were equipped with sterile jugular cannulae for intravenous administration of LPS. An abdominal laparotomy was performed, and a miniature strain gauge (RB Products, Madison, WI) was secured to the ventral surface of the antral portion of the stomach, in parallel with the circular smooth muscle (14, 15, 19). After the initial surgical preparations were complete, the animal was mounted in a stereotaxic frame in a nose-down orientation. The dorsal spinomedullary junction was exposed by resecting the dorsal cervical musculature, removing the occipital plate, and resecting both the dura mater and arachnoid meninges. Rats were randomly assigned to receive systemic injections of either 25 μg/kg or 1,000 μg/kg iv LPS and fourth ventricular applications of either TNFR:Fc or Fc fragment. One control group was surgically prepared as described above, received no intravenous LPS, and was subjected to repetitive ventricular applications of TNFR:Fc. An additional control group was subjected to the low dose of LPS (25 μg/kg iv) and received 40 μg iv TNFR:Fc according to the same delivery schedule as described below for the ventricular applications of TNFR:Fc. These two doses of systemic LPS were chosen because the 25 μg/kg dose was shown to evoke a significant c-Fos activation of NST neurons in our previous study (13) and does not produce hypotensive side effects. The higher LPS dose (1,000 μg/kg) is frequently cited in the literature as a dose appropriate to elicit a variety of systemic pathophysiological illness effects [e.g., hypotension and fever (39)] and had been used in our earlier studies (15) to elicit suppression of centrally stimulated gastric motility.

Experimental design. Gastric motility was continuously monitored once the animal was secured in the stereotaxic frame. A minimum of 20 min of baseline gastric motility was collected before the intravenous administration of LPS (1,000 μg/kg, n = 8; 25 μg/kg, n = 12; 0 μg/kg, n = 6) at time 0. The total volume of intravenous LPS injections was ~0.3 ml; it was delivered over 60–90 s. Simultaneous to the intravenous LPS injection and every 10 min for the next 90 min, 2 μl of either TNFR:Fc (2 μg/μl iv) or Fc fragment (2 μg/μl) were applied to the exposed floor of the fourth ventricle or TNFR:Fc (4 μg/0.1 ml iv) was injected. This intracerebroventricular dose of TNFR:Fc has been shown to neutralize TNF-α in the central nervous system (CNS) in other models (34).

The rationale for this method of application of TNFR:Fc or Fc to the floor of the fourth ventricle is based on four factors. First, TNFR:Fc compound adsorbs circulating TNF-α and is not a TNF-α receptor antagonist per se. Therefore, as each molecule of TNFR:Fc binds with two molecules of TNF-α, this construct is no longer available to remove more of the TNF-α in the circulation and the receptor continues to be receptive. Second, given that the floor of the fourth ventricle is in the stream of flow of cerebrospinal fluid into the spinal cord, both free and bound TNFR:Fc were continually being washed through the ventricular system and needed to be reapplied. Third, although injections of TNFR:Fc into the neuropil of the DVC were contemplated, as explained above, multiple, repeated, and bilateral injections of the TNF-α adsorbant would have been required to guarantee neutralization of the large and incrementing flood of TNF-α generated in the systemic circulation after the LPS challenge (15, 35). Fourth, although maximal plasma concentrations of TNF-α occur at 60–90 min after LPS exposure, small amounts can be detected within 30 min (35). Thus TNFR:Fc (or Fc) applications were made for the full period of augmenting TNF-α production. Finally, a control group that received the low dose of intravenous LPS also received intravenous TNFR:Fc construct at the same dose as the ventricular TNFR:Fc group. The purpose of this experimental group was to control for the...
eventuality that the TNFR:Fc construct “spilled” from the ventricular area into the systemic circulation and its true site of action was peripheral.

Ninety minutes after LPS administration (i.e., when plasma levels of TNF-α are maximal), a central stimulant of gastric motility, TRH, was applied to the floor of the fourth ventricle (2 μl of 100 μM). TRH acts within the DVC by both activating vagal cholinergic efferent neurons in the DMN and inhibiting NST neurons that receive afferent input from the gut (3, 33). The result is a dramatic increase in gastric motility (14, 15, 28). Our previous studies (14) have demonstrated that femtomolar doses of TNF-α in the DVC are sufficient to completely override this TRH-mediated increase in gastric motility. Therefore, if endogenous TNF-α is acting at the DVC to affect this suppression of gastric motility, then the presence of TNFR:Fc should adsorb and inactivate it. In this case, a TRH-mediated increase in gastric motility should be observed. In contrast, the Fc fragment should not be able to neutralize the TNF-α; the TRH-mediated increase in gastric motility will be suppressed.

At the 90-min interval, a terminal blood sample was obtained via cardiac puncture immediately before euthanasia to determine the circulating levels of TNF-α in each animal. Plasma TNF-α levels were determined by ELISA.

Data collection and analysis. Gastric motility was continuously monitored via the miniature strain gauge connected to a Wheatstone bridge-based amplifier (14). Each strain gauge was calibrated before the experiment by hanging known masses (0.1–2.0 g) on the gauges at the points where they would be sutured to the stomach. Calibrated output from the strain gauge amplifier was directed to a Grass polygraph and to the analog-to-digital converter inputs of a waveform storage/analysis system (Datapac 2000; Laguna Hills, CA). Gastric motility data could be displayed in real time and digitized for subsequent analysis or graphic display.

Motility records were analyzed in 300-s epochs. Peak voltage data (i.e., maximal contractions) during the 300-s epoch being analyzed were determined. Maximal gastric contraction amplitudes were compared (i.e., post- vs. pre-TRH application to the floor of the fourth ventricle) and expressed as peak motility ratios. Thus each animal served as its own control; motility ratio values >1 represent an increase in motility in response to TRH exposure, whereas motility ratio values ≤1 represent no response to TRH stimulation. Motility ratios were used for statistical and graphic purposes. The duration of the central prokinetic TRH effect was also compared. Duration was quantified as the span of time in which the TRH-induced increase in motility produced gastric contractions at least twice as large as contractions that occurred during the basal period.

TNF-α assay and data analysis. Concentrations of plasma TNF-α were determined via a double-sandwich ELISA protocol as recommended by R&D Systems (Minneapolis, MN). All incubations were done at room temperature. Microwells were washed three times with wash buffer (i.e., PBS, pH 7.4, with 0.05% Tween 20) between each addition/incubation step. Briefly, microwells of 96-well immunoplates (Nunc; Fisher Scientific, Pittsburgh, PA) were precoated with monoclonal anti-rat TNF-α antibody (R&D Systems) in PBS and incubated overnight. Nonspecific binding was blocked with PBS containing 1% BSA. Fifty to one-hundred microliters of unknown sera samples or TNF-α standards (rat recombinant TNF-α; R&D Systems) that had been diluted by 1:2–1:100 with Tris-buffered saline (TBS, pH 7.3) plus 0.05% Tween 20 and 0.1% BSA was added to the microwells and incubated for 2 h. TNF-α was detected via 2-h incubation with biotinylated anti-rat TNF-α antibody (R&D Systems; diluted in TBS with 0.1% BSA), streptavidin horseradish peroxidase conjugate (Zymed, San Francisco, CA; with 20-min incubation), and a final 20-min incubation in the dark after the addition of K-Blue Max substrate (Neogen, Lexington, KY). The reaction was stopped by the addition of 1 M H2SO4. The optical absorbance of each well was read within 30 min using a microplate reader set to 450 nm. Absorbance values were converted to TNF-α concentrations by comparison with a simultaneously generated standard curve. The minimum and maximum limits of detection per well of this assay were 15–1,000 pg/ml.

Statistical analyses. Plasma levels of TNF-α in response to systemic administration of LPS (i.e., 0, 25, or 1,000 μg/kg body wt iv) across all six experimental groups were log transformed for normalization before statistical analysis (22). Normalized data were subjected to one-way overall ANOVA. Statistical significance was defined as an overall P < 0.05; selected Bonferroni multiple comparison posttests were applied.

Maximal gastric contraction amplitudes elicited by central TRH were compared with maximal basal gastric contractions immediately preceding application of TRH to the DVC (i.e., post- vs. pre-TRH application to the floor of the fourth ventricle) and expressed as peak motility ratios. Motility ratios of all five groups that had received systemic LPS exposure were evaluated by one-way ANOVA; overall P < 0.05 value was considered statistically significant and permitted Newman-Keuls post hoc comparisons. Thus we determined whether the ventriculally administered TNFR:Fc construct could adsorb and inactivate endogenous TNF-α effects in the DVC and allow TRH-induced increases in gastric motility. The duration of the TRH-induced increase in motility in the two groups that had received ventricular TNFR:Fc was analyzed by Student's t-tests.

The possibility existed that repetitive ventricular administration of TNFR:Fc might inherently potentiate the response to TRH induction of gastric motility. Therefore, the two groups that had received 0 μg/kg iv LPS and either TNFR:Fc or Fc fragment alone over the 90-min interval were compared in their responsiveness to TRH application to the DVC. Peak motility ratios elicited by TRH application to the DVC area of these two groups were analyzed by Student's t-tests.

Relationships between circulating levels of TNF-α and either the magnitude or duration of the motility response to TRH stimulation were evaluated using the Spearman rank correlation test (22).

RESULTS

Plasma levels of TNF-α. Circulating levels of TNF-α are undetectable by ELISA in normal, healthy, unchallenged animals (11, 13, 35). Intravenous challenges of either dose of LPS induced elevated plasma levels of TNF-α in Inactin-anesthetized rats (Fig. 1) at levels significantly above normal, healthy, but comparably surgically prepared animals (ANOVA overall P < 0.0001; Bonferroni selected posttests **P < 0.001). Although the variability in the response to systemic LPS challenge is larger at the higher dose of LPS, these animals had higher plasma levels of TNF-α compared with those that had received 25 μg/kg LPS (Fig. 1; *P < 0.01 Bonferroni selected posttests).

Adsorption of endogenous TNF-α within the fourth ventricle permits central TRH stimulation of gastric
motility. Raw motility records (e.g., Fig. 2) were quantitated for graphic and statistical purposes (Fig. 3). Maximal contraction indices (converted to peak voltage data) were determined for specific 300-s (5-min) epochs of time during the experiment. The maximal contraction indices from 0 to 5 min after TRH stimulation were compared with the basal level of each animal (i.e., peak motility ratios = post-TRH/pre-TRH).

Only fourth ventricular (intracerebroventricular) applications of the TNFR:Fc construct [to specifically adsorb the circulating TNF-α] within the DVC permitted TRH stimulation of DVC circuits that, ultimately, resulted in increased gastric motility (Fig. 3A; ANOVA overall $P < 0.0001$; $**P < 0.001$ Bonferroni selected posttests). Although the variability in the response to systemic LPS challenge is larger at the higher dose of LPS, these animals had higher plasma levels of TNF-α compared with those that received 25 μg/kg LPS ($^*P < 0.01$ Bonferroni selected posttests). It is important to note that intravenous administration of the same dose of TNFR:Fc did not effectively adsorb circulating TNF-α in the DVC area (i.e., peak motility ratios were not significantly different from 1.)

Only fourth ventricular (intracerebroventricular) applications of the TNFR:Fc construct [to specifically adsorb the circulating TNF-α] within the DVC permitted TRH stimulation of DVC circuits that, ultimately, resulted in increased gastric motility (Fig. 3A; ANOVA overall $P = 0.0006$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ Newman-Keuls multiple comparison posttest). Duration of the TRH-induced increased gastric motility was longer in the 25 μg/kg LPS challenged group compared with the 1,000 μg/kg LPS group ($t = 3.760, P = 0.0096$).

Fig. 1. Plasma levels of tumor necrosis factor-α (TNF-α) 90 min after intravenous lipopolysaccharide (LPS). Intravenous challenges of LPS (either 25 or 1,000 μg/kg iv) induced elevated plasma levels of TNF-α in Inactin-anesthetized rats significantly above normal, healthy, but comparably surgically prepared animals (plasma levels were log transformed for normalization before statistical analysis; ANOVA overall $P < 0.0001$; $**P < 0.001$ Bonferroni selected posttests). Although the variability in the response to systemic LPS challenge is larger at the higher dose of LPS, these animals had higher plasma levels of TNF-α compared with those that received 25 μg/kg LPS ($^*P < 0.01$ Bonferroni selected posttests). icv, Intracerebroventricular; iv, intravenous.

Fig. 2. Raw polygraph records of gastric motility recorded 90 min after administration of LPS (25 μg/kg body wt, iv). A: this animal had received LPS (iv) and continuous ventricular perfusion (icv) of the Fc fragment vehicle control. Ventricular application of TRH is unable to override the suppressive effects of endogenously produced TNF-α. B: this animal had received LPS (iv) and continuous ventricular perfusion of the TNFR:Fc absorbant construct (icv). Presumably, endogenously produced TNF-α is adsorbed as it passes through the ventricular flow and application of TRH to the floor of the 4th ventricle is able to maximally stimulate gastric motility.

Fig. 3. Maximal contraction indices (i.e., peak contractions converted to voltage data) over 5-min epochs of time were quantitated for statistical comparison. A: only intracerebroventricular TNFR:Fc restored the normal gastric motility response to TRH (icv). Intravenous administration of the TNFR:Fc icv dose was ineffective, as was the icv application of the Fc fragment alone. Maximal contraction indices before (−5–0 min) and after (0–5 min) TRH (icv) were compared for each individual rat. Thus each animal served as its own control. Additionally, the increase in gastric contraction indices was greater in the group that demonstrated the lower plasma level of TNF-α (i.e., 25 μg/kg LPS vs. 1,000 μg/kg LPS). ANOVA overall $P = 0.0006$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ Newman-Keuls multiple comparison posttest. B: duration of the TRH-induced increased gastric motility was longer in the 25 μg/kg LPS challenged group compared with the 1,000 μg/kg LPS group ($t = 3.760, P = 0.0096$).
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Fig. 4. The possibility existed that repetitive ventricular administration of TNFR:Fc might inherently potentiate the response to TRH induction of gastric motility. These two groups received 0 μg/kg iv LPS and either TNFR:Fc or Fc fragment alone over the 90-min interval. Responsiveness to TRH application to the dorsal vagal complex (DVC) was evaluated as peak motility ratios and analyzed by Student’s t-tests. Repetitive exposure of the DVC to TNFR:Fc alone did not potentiate the effectiveness of central TRH-induced increases in gastric motility (t = 0.51; P = 0.64).

DISCUSSION

Our previous studies (15) showed that endogenous production of TNF-α after systemic LPS challenge is associated with suppression of centrally stimulated gastric motility. The present experiment used the TNFR:Fc construct to specifically adsorb and inactivate TNF-α within the brain stem perfusion area to determine whether TNF-α action, within the medullary brain stem, is the causative agent for the previous observations.

In this study, all animals challenged with intravenous LPS demonstrated elevated plasma levels of TNF-α. Under normal circumstances (i.e., no LPS exposure), intracerebroventricular application of TRH evokes a large, prolonged, and vagally mediated increase in gastric contractions (14, 15, 28). Endogenous production of TNF-α after systemic LPS challenge suppresses this centrally stimulated gastric motility (15). In the present study, continuous perfusion of the floor of the fourth ventricle with TNFR:Fc after systemic LPS challenge disinhibited the centrally commanded (i.e., TRH) increase in gastric motility. That is, TNFR:Fc specifically adsorbed and inactivated circulating TNF-α in this medullary area and permitted the TRH stimulation of the DVC to increase gastric motility. Thus these results support the hypothesis that circulating TNF-α can act within the brain stem DVC to inhibit gastric function. Furthermore, the specific removal of TNF-α from the vicinity of vagovagal reflex control circuitry is sufficient to restore a normal CNS-generated increase in motility.

Therapeutic regimens that cause reductions in TNF-α production are well recognized for their anti-inflammatory and gastric prokinetic effects. Doses of glucocorticoids sufficient to cause immune suppression through inhibition of TNF-α production can relieve nausea and gastric stasis associated with cancer chemotherapy, which, by itself, can evoke very large increases in TNF-α production (4, 9). Furthermore, as we suggested in an earlier paper (15), it is very likely that the antinausea properties of thalidomide in emesis gravidarum (morning sickness) are due to the effect of the drug to retard the synthesis of TNF-α (see Ref. 15). TNF-α is highly pleiotropic and pleuripotential (6, 32) and the case of thalidomide points out the potential for the development of serious unintended consequences of the systemic manipulation of the cytokine. Although thalidomide is safe and effective in its role as an anti-inflammatory, e.g., treatment of leprosy and lupus (39) and antinausea compound (25), thalidomide used during early pregnancy reveals severe teratogenic effects on fetal limb development due to the role of TNF-α as a regulator of angiogenesis and limb bud development (38).

Gastric stasis, and possibly the nausea, associated with the many disease processes that involve the production of TNF-α may, at least partially, be explained by action of the cytokine directly on neurons of the dorsal vagal complex. However, elimination of these pathophysiological consequences of TNF-α action by blocking TNF-α action is a course that must be taken with caution.

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