Roles of IL-1 and TNF in the decreased ileal muscle contractility induced by lipopolysaccharide

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tumor necrosis factor; interleukin-1; tumor necrosis factor binding protein; interleukin-1 receptor antagonist; soluble tumor necrosis factor receptor; nitric oxide; inducible nitric oxide synthase; nitric oxide synthase activity; intestinal transit; endotoxin; ileal smooth muscle

ALTHOUGH SEPSIS is the most common cause of death in medical intensive care units, with a mortality of ~50%, its pathophysiology is only incompletely understood. The syndrome of sepsis is a systemic inflammatory response to invading microorganisms. This response is now believed to be the result of a cascade of mediators: microbial toxins, such as lipopolysaccharide (LPS) endotoxin of gram-negative bacteria, cause the release by the host of potent proinflammatory mediators, which, in turn, are largely responsible for the cardiovascular derangement, shock, multiple organ failure, and death (26, 28). The proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF) are principal among the host mediators.

Gastrointestinal stasis, or ileus, commonly accompanies sepsis and multiorgan dysfunction in critically ill patients and may contribute to their pathophysiology. The mechanism of this impairment of transit during sepsis is unclear but may be associated with impaired contractile function of gastrointestinal smooth muscle. The administration of LPS has been shown to result in a depression in contractility of skeletal (15), cardiac (3, 33), and smooth muscle, especially vascular smooth muscle (22, 32), in several animal models. In a previous study, we demonstrated that LPS administration to rats resulted in a decrease in contractility of ileal longitudinal smooth muscle (40). The reduction in contractility was associated with an upregulation of the inducible isoform of nitric oxide synthase (NOS II) and could be reversed by addition of a competitive inhibitor of NOS.

The current study was designed to test the hypothesis that the reduction in contractility and the upregulation of NOS activity induced by LPS are mediated by TNF and IL-1. We studied the roles of these two cytokines by pretreating the animals with their respective cytokine antagonists, TNF binding protein (TNFbp) and IL-1 receptor antagonist (IL-1ra), which specifically inhibit their action.

METHODS

Cytokine antagonists. TNFbp and IL-1ra were gifts of Synergen (Boulder, CO). Production of the TNFbp has been described in detail by Su et al. (35). Soluble TNF receptor I was produced by the introduction of a synthetically constructed gene encoding the free extracellular domain of human TNF receptor I into an Escherichia coli expression vector. The recombinant protein contains an additional amino terminal methionine and an asparagine/cysteine substitution (amino acid 105) to allow the attachment of a polyethylene glycol (PEG) linker. After the soluble TNF receptor I monomer was extracted and purified, a dimeric TNFbp was synthesized by covalent attachment of two soluble TNF receptor I monomer molecules to a bifunctional 20-kDa PEG group via cysteine residue 105 of the protein. The resulting TNFbp dimer has a molecular mass of ~56 kDa. It was dissolved in neutral PBS, and before administration it was diluted to a concentration of 1.5 mg TNFbp/ml. Pharmacokinetic studies indicate that the efficacy (minimal effective dose) of TNFbp dimer in animal models occurs at doses ranging from 0.3 to 5 mg/kg body wt administered every other day (35).

A nonylcylsylated form of human IL-1ra has been isolated from human monocytes in conditioned medium (16) and produced by recombinant DNA technology in E. coli (8).
Recombinant human IL-1ra is identical to the naturally occurring nonglycosylated human form of IL-1ra with the exception of the addition of one N-terminal methionine (12). It was formulated at a concentration of 100 mg IL-1ra/ml in a salt solution (CSE vehicle) containing 10 mM dithiothreitol, 140 mM sodium chloride, and 0.5 mM EDTA, at pH 6.5 (31).

Protocols. Two experimental protocols were followed: the first was designed to test the ability of the combined administration of TNFbp and IL-1ra to attenuate the effects of LPS administration; the second was designed to test the effects of each cytokine antagonist separately. To complete the first protocol, male Sprague-Dawley rats, 275–300 g, were divided into four groups of six animals each and were fasted overnight. One group (TNFbp + IL-1ra + LPS) was injected with TNFbp (1.5 mg/kg iv) plus IL-1ra (100 mg/kg sc) 1 h before [time (t) = −1 h] an intraperitoneal injection (at t = 0 h) of LPS from E. coli (serotype 0111:B4; 20 mg/kg body wt; Sigma Chemical, St. Louis, MO). The second group (TNFbp + IL-1ra + saline) was similarly injected with both cytokine antagonists at t = −1 h but received at t = 0 h an intraperitoneal injection of an equal volume of normal saline, the vehicle for LPS. The third group (vehicles + LPS) was injected at t = −1 h with the respective vehicle for the two cytokine antagonists followed with LPS at t = 0 h. The vehicle for IL-1ra was CSE buffer. The vehicle for TNFbp was neutral PBS. The fourth group (vehicles + saline), which served as the control group, was injected with cytokine antagonist vehicles at t = −1 h and with saline at t = 0 h. Because the effective half-lives of TNFbp and IL-1ra administered in this manner are ~30 and 6 h, respectively (Synergen, personal communication), a second injection of either IL-1ra or its vehicle was administered at t = +2 h. At t = +5 h after LPS or saline injection, the animals were killed, and full-thickness sections of ileum were taken from each animal. These sections were taken such that paired determinations of contractility and NOS activity could be determined for each animal.

The second protocol was similar to the first except that it tested the effects of single rather than combined cytokine antagonist treatment. Four additional groups of six male Sprague-Dawley rats, 275–300 g, were used. After an overnight fast, a single group (IL-1ra + LPS) was treated with IL-1ra followed in 1 h with LPS, a second group (TNFbp + LPS) was treated with TNFbp followed in 1 h with LPS, a third group (vehicles + LPS) was treated with vehicle followed in 1 h with LPS, and a fourth group (vehicles + saline) was treated with vehicle followed in 1 h with saline. As above, the groups that received IL-1ra or its vehicle received a second injection of the same at t = +2 h. At t = +5 h after LPS or saline injection, full-thickness sections of ileum were taken from each animal.

For the contractility studies, strips of longitudinal muscle ~1 cm long were peeled from segments of ileum that were to be analyzed for NOS activity were frozen immediately after recovery from the animals.

Ileal muscle contractility. Strips, two from each segment, were mounted in 10-ml organ baths filled with Krebs-Ringer solution (in mM: 103 NaCl, 4.7 KCl, 2.5 CaCl₂, 25 NaHCO₃, 1.1 NaH₂PO₄, and 15 glucose). The solution was gassed with 5% CO₂-95% O₂. Isometric force was monitored by an external force displacement transducer (Grass FT.03; Grass Instrument, Quincy, MA) connected to a Beckman R411 recorder (Beckman Instruments, Electronic Instrument Division, Schiller Park, IL; see Ref. 40). Each strip was allowed to equilibrate for at least 20 min, and then 10⁻² M carbamylcholine chloride [carbachol (CCh); Sigma Chemical] was added. After peak active force was reached, the bath was washed with fresh solution and allowed to stand for 20 min. The force of contraction produced and allowed to stand for 20 min. The force of contraction produced and allowed to stand for 20 min. The force of contraction produced and allowed to stand for 20 min. The force of contraction produced and allowed to stand for 20 min.
induced decrease in contractility, but cytokine antagonist treatment itself (TNFbp + IL-1ra + saline) had no effect \( P = \text{not significant (NS)} \) on the contractility of strips from healthy rats.

Figure 2 shows the maximum stress developed by the strips of rat ileal longitudinal smooth muscle in response to \( \text{CCh} \) (10\(^{-2}\) M) for the four groups in the first protocol. The control group (vehicles + saline) had a maximum stress of 508 ± 55 g/cm\(^2\) (mean ± SE), which was not affected by cytokine antagonist treatment itself (TNFbp + IL-1ra + saline: 498 ± 23 g/cm\(^2\); \( P = \text{NS} \)). LPS treatment (vehicles + LPS) significantly decreased the maximum stress to 355 ± 33 g/cm\(^2\) (\( P < 0.05 \)). Cytokine antagonist treatment before LPS (TNFbp + IL-1ra + LPS) prevented the LPS-induced decrease in maximum stress (486 ± 48 vs. 355 ± 33 g/cm\(^2\); \( P < 0.05 \)), yielding a maximum stress value not different from control (\( P = \text{NS} \)).

Figure 3 shows the \( EC_{50} \) values, derived from the contractility curves in Fig. 1, for the four groups in the first protocol. For the control group (vehicles + saline), \( EC_{50} \) was 3.46 ± 10\(^{-2}\) M (log\(_{10}\) units, -7.46 ± 0.11, mean ± SE). \( EC_{50} \) was not significantly affected by LPS or cytokine antagonist treatment.

Figure 4 shows the maximum stress developed by the strips of rat ileal longitudinal smooth muscle in response to \( \text{CCh} \) (10\(^{-2}\) M) for the four groups in the second protocol. These values were derived from full dose-response curves (10\(^{-8}\) to 10\(^{-4}\) M \( \text{CCh} \)) similar to those of Fig. 1. The control group (vehicles + saline) had a maximum stress of 498 ± 50 g/cm\(^2\). As in the first
protocol, LPS (vehicles + LPS) significantly reduced the maximum stress (386 ± 16 g/cm²; P < 0.05). This LPS-induced reduction in maximum stress was prevented by pretreatment with TNFbp alone (TNFbp + LPS; 491 ± 24 g/cm²) and ameliorated by pretreatment with IL-1ra alone (IL-1ra + LPS; 431 ± 24 g/cm²). The maximum stress for pretreatment with TNFbp alone was significantly greater than that for LPS (P < 0.05) and not different from that of control (P = NS). Maximum stress for IL-1ra pretreatment was intermediate between the control and LPS values, but these differences did not reach statistical significance. As observed in the first protocol, the EC₅₀ for CCh did not differ among the four groups in the second protocol.

NOS II activity. The NOS activity that was calcium independent (NOS II activity) and inhibited by L-NAME is presented in Fig. 5. Activity was detected in ilea from control (vehicles + saline). Cytokine antagonist treatment by itself (TNFbp + IL-1ra + saline) tended to decrease this basal activity, but the change did not reach significance. LPS (vehicles + LPS) increased ileal NOS II activity by >10-fold (P < 0.01). This marked increase in NOS II activity by LPS was largely prevented by cytokine antagonist pretreatment (TNFbp + IL-1ra + LPS), which resulted in NOS II activity that was significantly less than that for LPS treatment (P < 0.01) and not significantly different from control (P = NS).

Correlation between NOS II activity and maximum stress. In the first protocol, both NOS II activity and maximum stress were determined in the same animals. A plot of maximum stress as a function of NOS II activity for these animals is shown in Fig. 6. There was a statistically significant (P < 0.03) inverse relationship between NOS II activity and maximum stress.

Fig. 5. Inducible nitric oxide synthase (NOS II) activity, assayed by calcium-independent arginine-to-citrulline conversion rates, in full-thickness sections of rat ileum for the four treatment groups shown. Compared with control, LPS markedly increased NOS II activity by ~10-fold (***P < 0.001). Pretreatment with TNFbp + IL-1ra prevented this increase in LPS-treated rats (††P < 0.01 compared with vehicles + LPS) but had no significant effect in healthy, saline-treated rats. See text and Fig. 1 legend for details.

Fig. 6. Relationship between maximum stress generated by carbachol and NOS II activity for rat ileal tissue for the four treatment groups shown. Maximum stress was negatively correlated with NOS II activity. See text and Fig. 1 legend for details.

DISCUSSION

Prolonged gastrointestinal stasis, or ileus, is a common complication of sepsis and, in fact, may perhaps be considered a single-organ example of the multiorgan dysfunction that characterizes severe sepsis. We and others have shown that a decrease in transit allows the overgrowth of bacteria within the lumen of the small intestine and eventual translocation of bacteria from the gut into the regional lymph nodes (23, 24, 30). These observations indicate that gut stasis may be not only a result of sepsis but also a cause of sepsis, particularly of persistent or recurrent sepsis, by virtue of such a “vicious cycle.” The mechanisms of the impairment of gastrointestinal transit are not clearly defined but may be associated with impairment in the contractile function of smooth muscle.

We have previously demonstrated that LPS, given in vivo to healthy rats, causes a decrease in the contractility of ileal smooth muscle, measured in vitro (40). In addition, we showed that this decrease in contractility is associated with an LPS-induced upregulation of NOS II mRNA, immunoreactivity, and enzymatic activity in the mucosal and muscle layers of the small intestine (40). We also showed that the impaired contractility could be reversed by L-NAME, a competitive inhibitor of NOS. Similar results have been reported for vascular smooth muscle (32).

To investigate the mechanisms of LPS-induced impairment of gut smooth muscle contractile function, we took advantage of the large body of information on the mechanisms of cardiovascular impairment in sepsis. The cardiovascular derangement in severe sepsis is characterized by impairment of both cardiac and vascular smooth muscle function. When severe, these impairments result in vasodilatation, hypotension, and hypoperfusion that are refractory to intravenous infusion of fluids and to vasoconstrictor and inotropic agents (25). If uncorrected, this cardiovascular compromise leads ultimately to multiorgan failure and death (26, 28). Extensive evidence now indicates that the cardiovascular derangement in sepsis is the result of a
complex cascade of mediators. This cascade begins with a nidus of infection-releasing organisms or their products, such as LPS, which in turn cause the release by the host of numerous proinflammatory mediators. Principal among the proinflammatory mediators are TNF and IL-1. We (19–21) and others (2, 10, 18, 29, 37, 38) have shown that the cardiovascular derangement caused by LPS, TNF, or IL-1 is largely mediated by the induction of NOS II, resulting in excessive production of nitric oxide.

The discoveries of the natural occurrence of endogenous antagonists to TNF and IL-1 (6, 9, 27) provide a unique opportunity to study the roles of these cytokines in the LPS-induced derangement in gastrointestinal motility. The biological actions of TNF are mediated by two distinct cell surface receptors, a 55-kDa type I (p55) receptor and a 75-kDa type II (p75) receptor. Soluble forms of these receptors are shed by proteolytic cleavage (serine protease) of their extracellular domains. Soluble TNF receptors inhibit the action of TNF by the formation of high-affinity complexes, thereby preventing TNF from binding to target cell membrane receptors (6, 27). Soluble TNF receptors are found in the circulation of healthy individuals. Endotoxemia induces a severalfold increase in these receptors (34, 39), an effect mediated largely by the increase in TNF itself (17). In several animal models of sepsis and inflammation, administration of the soluble p55 TNF receptor has reduced inflammation and prolonged survival (6, 27). In other approaches, each of these soluble TNF receptors has been fused with the Fc portion of IgG1, forming chimeric “fusion proteins.” In human patients with sepsis, a phase II trial of the p55–IgG1 construct had favorable results (the results of a phase III trial are pending; see Ref. 1). A phase III trial of the p75–IgG1 construct (11) was disappointing, but the difference has been attributed in part to the more stable kinetics (much slower exchange rate) of the p55 fusion protein (1).

IL-1ra was recently identified as the third member of the IL-1 gene family, joining IL-1α and IL-1β (5). IL-1ra binds to the type I cell-surface IL-1 receptor with an affinity similar to that of IL-1α and IL-1β, but it has no agonist effect, even at high concentrations. Thus IL-1ra functions as a naturally occurring, specific antagonist of both IL-1α and IL-1β by blocking the binding and the biological activities of these two cytokines (5). Blood levels of IL-1ra are substantially increased by endotoxin, IL-1 itself, or TNF (5). In a number of animal models of sepsis, early administration of IL-1ra (before significant IL-1 synthesis and release) has been shown to decrease both mortality and morbidity (5, 7) and to attenuate the LPS-mediated induction of NOS II (36). Although the results of an initial phase III trial of IL-1ra in human patients were encouraging (12), a subsequent phase III trial was disappointing (6), perhaps due in part to the relatively late administration of IL-1ra (up to 3 days after the onset of the infection causing the sepsis).

In the present study, we used specific formulations of the two cytokine antagonists, TNFbp and IL-1ra, to study the roles of their corresponding proinflammatory cytokines in mediating the LPS-induced decrease in ileal smooth muscle contractility and increase in NOS II activity.

The present study confirms our previous findings (40) that LPS causes a decrease in the contractility of ileal smooth muscle and an increase in ileal NOS II activity. As previously, we also showed that the reduction in contractility was characterized by a decrease in maximum response but with no significant change in the concentration of CCh that elicited 50% of that maximum response, that is, LPS did not result in a rightward shift in the dose-response curves.

In our earlier study (40), we found that 20 mg/kg LPS induced an increase in NOS II mRNA and immunoreactivity (protein) that could be detected both in full-thickness samples of ileum and samples of longitudinal muscle plus serosa. Furthermore, for each individual animal, the magnitude of the increases in the muscle layer appeared to correlate with that in full-thickness samples even though absolute levels in the muscle were lower. Only qualitative changes were assessed because the methods used to determine the increases in mRNA and immunoreactivity are not quantitative. To be quantitative, NOS II enzymatic activity was assayed in the present study. Although we successfully determined NOS II activity in full-thickness samples of ileum, our attempts to determine NOS II activity in samples of longitudinal muscle plus serosa were not successful, presumably due to the lower abundance of the enzyme in the muscle layer. In the present study, our goal of correlating muscle contractility with NOS II activity (see Fig. 6) required quantitative data. Thus we determined NOS II activity quantitatively in full-thickness samples. It is possible that the cytokine antagonists selectively inhibited the upregulation of NOS II only in the nonmuscle layers of the intestine. However, we are aware of no studies showing a selective action based on anatomical location. Thus it is more likely that the changes in NOS II activity monitored in the combined layers of the intestine reflected the changes in NOS II activity in the longitudinal muscle layer.

The principal new findings in the present study were that both the decrease in ileal muscle contractility and the increase in NOS II activity were largely prevented by pretreatment with cytokine antagonists that inhibit the action of TNF and IL-1. At the doses and time intervals we employed, TNFbp alone was more effective than was IL-1ra alone in preventing the decrease in contractility, indicating that TNF may be the more dominant mediator. However, full dose-response and time-response relationships need to be elucidated before any primacy can be assigned. An additional new finding was that the level of contractility was negatively correlated with the level of NOS II activity. Overall, these results suggest that LPS impairs ileal smooth muscle contractility predominantly by stimulating the production of TNF and IL-1, which in turn act by induction of ileal NOS II, which produces increased levels of the potent smooth muscle relaxant nitric oxide. This suggested cascade of mediators is consistent...
with that proposed for the pathogenesis of sepsis in general (26, 28).

The present study provides evidence that the LPS-induced derangement in gut smooth muscle function, similar to the LPS-induced derangements in cardiac and vascular smooth muscle functions, is the result of the proinflammatory cascade that includes TNF, IL-1, and ultimately nitric oxide. Further exploration of this proinflammatory cascade may provide new insight into the pathogenesis of the prolonged gastrointestinal status that often complicates the course of patients with sepsis and may suggest novel therapeutic approaches.

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