Lipopolysaccharide-induced changes in mesenteric afferent sensitivity of rat jejunum in vitro: role of prostaglandins

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Wang, B., J. Glatzle, M. H. Mueller, M. Kreis, P. Enck, and D. Grundy. Lipopolysaccharide-induced changes in mesenteric afferent sensitivity of rat jejunum in vitro: role of prostaglandins. Am J Physiol Gastrointest Liver Physiol 289: G254–G260, 2005. First published March 24, 2005; doi:10.1152/ajpgi.00329.2004.—Bacterial translocation across the intestinal mucosal barrier leads to a macrophage-mediated inflammatory response, visceral hyperalgesia, and ileus. Our aim was to examine how mediators released into mesenteric lymph following LPS treatment influence intestinal afferent sensitivity and the role played by prostanoids in any sensitization. Intestinal lymph was collected from awake rats following treatment with either saline or LPS (5 mg/kg ip). Extracellular multiunit afferent recordings were made from paravascular mesenteric nerve bundles supplying the rat jejunum in vitro following arterial administration of control lymph, LPS lymph, and LPS. Mesenteric afferent discharge increased significantly after LPS lymph compared with control lymph. Peak discharge occurred within 2 min and remained elevated for 5 to 8 min. This response was attenuated by pretreatment with naproxen (10 μM), and restored upon addition of prostaglandin E2 (5 μM) in the presence of naproxen, but AH6809 (5 μM), an EP1/EP2 receptor(s) antagonist, failed to decrease the magnitude of LPS lymph-induced response. LPS itself also stimulated mesenteric afferent discharge but was unaffected by naproxen. TNF-α was significantly increased in LPS lymph compared with control lymph (1.583 ± 197 vs. 169 ± 38 pg/ml, P < 0.01) but exogenous TNF-α failed to evoke any afferent nerve discharge. We concluded that inflammatory mediators released from the gut into mesenteric lymph during endotoxemia have a profound effect on afferent discharge. These mediators influence afferent firing via the release of local prostanoids.

cytokines; lymph; hypersensitivity

LPS from Gram-negative bacteria, also known as endotoxin, can trigger a macrophage-driven cytokine cascade that is referred to as an acute-phase response. This drives a local inflammatory reaction and generates behavioral responses known as sickness behavior that include fever, anorexia, and hyperalgesia (21). In animal models, systemic LPS can cause ileus (8), rectal hypersensitivity (5), and also produces a profound increase in the afferent discharge emanating from the bowel wall (19). The macrophage-driven cytokine cascade following LPS administration gives rise to an increase in circulating IL-1β and TNF-α (7). These cytokines orchestrate both the local inflammatory response and the central nervous system, consequences that are manifested as illness behavior. These central nervous system consequences appear to involve both direct effects of circulating cytokines and activation of afferent inputs to the central circuits that regulate temperature, feeding behavior and pain modulation (7, 21). The mechanisms underlying these changes in afferent sensitivity have not been determined although recent work from our laboratory suggests that prostanoids may play a pivotal role in the sensitization process (19, 23). However, changes in afferent sensitivity following systemic LPS can involve the generation of mediators locally in the gut wall, but also elsewhere in the body, which reach the gut via the circulation. Thus the contribution of local vs. systemic mediators to LPS-driven hyperalgesia needs to be determined.

Mediators released from the gut wall during sepsis appear in the lymph and can mediate inhibition of gastrointestinal motility (10). Harvesting lymph from LPS-treated animals therefore represents a method of assessing the contribution of such systemic mediators to the LPS-driven hyperalgesia. In the present study, we harvested lymph from LPS-treated animals and control animals and compared the effect of this lymph with the effect of LPS itself on the activation of afferents supplying the rat jejunum. With this model, it has been possible to distinguish for the first time the direct effects of LPS on afferent nerve discharge vs. indirect effects triggered by gut-derived mediators originally released by LPS challenge. Here we demonstrate the contribution of prostanoids to these direct and indirect effects on afferent firing.

MATERIALS AND METHODS

Animals

Forty-four male Sprague-Dawley rats (300–350 g) purchased from Charles River (Sulzfeld, Germany) were fed regular laboratory chow with free access to water and housed under controlled conditions with...
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a 12:12-h light-dark cycle. Institutional guidelines for the care and use of laboratory animals were followed throughout the study and approved by the institutional review board at the University of Tuebingen.

**Mesenteric Lymph Collection**

**Surgical preparation.** Rats \((n = 8)\) were fasted overnight but allowed water ad libitum before surgery. Mesenteric lymph was collected as described previously \((9, 10)\). Briefly, rats were anesthetized with methohexital sodium \((60 \text{ mg/kg ip}; \text{Brevital; Johns Pharma, St. Louis, MO})\) and the mesenteric lymph duct, draining the area of the superior mesenteric artery, was cannulated with a polyvinyl tube \((\text{medical grade; 0.50 mm ID, 0.80 mm OD; Dural Plastics and Engineering, Dural, NSW, Australia})\) fixed in place with a drop of ethyl cyanoacrylate glue \((\text{Krazy Glue; Elmer’s Products, Columbus, OH})\) and exteriorized through a surgical incision in the right flank. A second cannula \((\text{silicone elastomer; 1 mm ID, 2.15 mm OD})\) was passed through the fundus of the stomach into the duodenum and secured with a purse-string suture. After surgery, rats were placed in Bollin cages and a glucose-saline solution \((0.2 \text{ M glucose, 145 mM NaCl and 4 mM KC})\) was infused continuously through the duodenal cannula at a rate of 3 ml/h to equalize fluid loss via the lymph. After a 24-h recovery period, mesenteric lymph was collected for 12 h following intraperitoneal administration of either normal saline as control or LPS \((5 \text{ mg/kg, from Escherichia coli, Serotype 0111:B4})\). Lymph was collected in ice-chilled tubes, centrifuged, and an aliquot was taken for the cytokine assays. The lymph collected from each of the two groups of animals was pooled and frozen at \(-80^\circ\text{C}\) until used. Animals were killed by anesthetic overdose at the end of this procedure.

**Determination of TNF-α and IL-1β in lymph.** Concentration of TNF-α and IL-1β in mesenteric lymph taken from each animal were determined using standard rat TNF-α and IL-1β Immunoassay Kits \((\text{Biosource, Camarillo, CA})\) with sensitivity < 3 pg/ml.

**Mesenteric Afferent Nerve Recordings**

Rats \((n = 36)\) were anesthetized with pentobarbitone sodium \((60 \text{ mg/kg ip})\) and a midline laparotomy was performed. The terminal jejunal and its arterial supply were identified. A branching section of artery with a clear projection to a segment of the jejunum was chosen, and the mesenteric arcade was pulled through an aperture leading into a separate recording chamber. The mesenteric arcade was cannulated and connected with a pump to permit intravascular perfusion with Krebs buffer \((0.15 \text{ ml/min})\). To prevent accumulation of fluid in the recording chamber, the venous effluent was allowed to drain into the organ chamber by puncturing the small veins draining the segment. The aperture linking the two compartments was then sealed with Vaseline petroleum jelly, and the recording compartment was filled with heavy liquid paraffin. The preparation was allowed to warm slowly, reaching a working temperature of \(\sim 34^\circ\text{C}\) before mesenteric nerve recording was obtained.

Under a stereo microscope, one of the two paravascular nerve bundles was exposed between the artery and vein. The surrounding connective tissue was carefully removed, and the nerve bundle was wrapped around one arm of a bipolar platinum recording electrode. One piece of connective tissue was attached to the second electrode. The electrodes were connected to an Neurolog headstage \((\text{Digitimer NL 100})\), and the signal was amplified \((\text{NL 104, 20,000})\) and filtered \((\text{NL 125 band width 100–1,000 Hz})\) then relayed to a spike processor \((\text{Digitimer D130})\) to allow discrimination of action potentials from noise using manually set amplitude and polarity window. Whole nerve activity was continually monitored as spike discharge \([\text{impulses (imp/s)] and stored together with the raw nerve signal and output from the pressure transducer on a computer running Spike-2 software (Cambridge Electronic Design).}

**Experimental Protocols**

Experiments were performed on preparations in which baseline afferent discharge was maintained for at least 10 min and a robust nerve response to a submaximal dose of intra-arterial 5-HT \((47 \mu\text{g/ml, 0.3 ml})\) could be evoked. Preparations that failed to respond to 5-HT were assumed to be inadequately perfused and were excluded from the study. A 15-min recovery was allowed before beginning the subsequent protocols.

Three groups of experiments were performed in this study. In the first, Krebs solution, control lymph, LPS lymph, and then LPS \((3 \text{ mg/ml})\) were infused intra-arterially, each over a 2-min period and separated by an interval of 15 min \((n = 8)\). In pilot experiments \((n = 3)\), LPS at this concentration was shown to evoke a robust mesenteric afferent response and was used as a standard stimulus at the end of each experiment. The time course and magnitude of the LPS response was comparable when given alone or following LPS lymph, indicating that the response to LPS was not conditioned by prior administration of LPS lymph.

The second series of experiments examined the involvement of prostaglandin synthesis in LPS-induced responses. In these experiments LPS lymph and then LPS were infused as above, following pretreatment with naproxen in the perfusing fluid \((10 \mu\text{M, } n = 6)\), naproxen \((10 \mu\text{M})\) plus PGE\(_2\) \((5 \mu\text{M, } n = 5)\), or AH6809 \((5 \mu\text{M, } n = 6)\). All treatments began 10 min before beginning the recording of baseline discharge.

In the final series of experiments, TNF-α was administered either intra-arterially \((0.3 \text{ ml, and 10 ng/ml, } n = 4)\) or in the perfusing fluid at a final concentration of 10 ng/ml \((n = 4)\) to test the influence of this inflammatory cytokine on afferent firing.

**Materials**

All salts used for the Krebs buffer were obtained from Sigma \((\text{St. Louis, MO})\) or Merck and were of AnalaR grade. LPS, naproxen \((\text{[–]-sodium 6-methoxy-α-methyl-2-naph-thaleneacetae})\), PGE\(_2\) \((\text{[5Z, 11α, 13E, 15S]-11, 15-dihydroxy-9-oxoproposta-5, 13-dienoic acid})\), and AH6809 \((\text{6-Isoproxy-9-oxoxanthene-2-carboxylic acid})\) were obtained from Sigma. Recombinant rat TNF-α \((\text{Biosource})\) was dissolved in 0.1% BSA in buffered saline to a concentration of 10 ng/ml and stored at \(-86^\circ\text{C}\) until use. PGE\(_2\) \((1 \text{ mg})\) was dissolved in 0.1 ml of absolute ethanol and then 0.9 ml of a 2% solution of sodium carbonate was added to make a stock solution of 1 mg/ml. AH6809 was dissolved in dimethyl sulphoxide \((6 \text{ mM})\) and frozen at \(-20^\circ\text{C}\) before use.

**Data Analysis and Statistics**

All multiunit recordings showed a continuous pattern of on-going discharge in the absence of any intentional stimulation. Baseline discharge was determined during the 5-min period before stimulation. Increases in mesenteric discharge above this baseline following treatment are expressed as impulses per second and presented as means ± SE. These increases in discharge under the various experimental conditions were evaluated using ANOVA followed by post hoc Dunnett’s multiple comparison test using the software package of

\[a \cdot b, c \text{ and } d \]
GraphPad Prism 3.02 (San Diego, CA). Biochemical data derived from samples of control and LPS lymph were compared using an unpaired Students’ t-test. Values of P < 0.05 were considered significant.

RESULTS

Effects of LPS Lymph and LPS on Mesenteric Afferent Discharge

Whole-nerve discharge did not change following the infusion of Krebs buffer; however, both LPS lymph and LPS evoked a significant increase in whole-nerve discharge (Fig. 1). In every case following LPS (n = 11) and in three of eight cases following LPS lymph, this increase in discharge was accompanied by a reduction in jejunal contractile activity (Fig. 1). LPS induced a more sustained inhibition in the contractile activity than LPS lymph (see bottom trace in Fig. 1 for example). Mesenteric discharge peaked around 100 s following the onset of infusion and the magnitude of this peak was similar for LPS lymph and LPS (Figs. 2 and 3). The time course of recovery of mesenteric afferent firing was also similar for LPS lymph and LPS (Fig. 2). The administration of control lymph did not result in a significant increase in mesenteric afferent discharge (n = 8) although in two individual cases there was a modest increase in afferent discharge (Fig. 3) and in one of these, there was an associated reduction in spontaneous contractile activity.

Effect of TNF-α

The level of TNF-α in mesenteric lymph was almost 10 times higher in animals treated with LPS compared with saline-treated controls (1,583 ± 197 vs. 169 ± 38 pg/ml, P < 0.001, see Fig. 4). In contrast, the concentration of IL-1β was similar in control and LPS lymph (64 ± 15 vs. 60 ± 8 pg/ml).

Effect of Naproxen, PGE2, and AH6809

The involvement of prostanoids in the response to LPS and LPS lymph was investigated by adding the cyclooxygenase (COX) inhibitor naproxen (10 μM) to the bathing medium. Baseline discharge in tissue treated with naproxen was 5.4 ± 1.3 imp/s (P > 0.05, vs. control group 12.4 ± 3.6 imp/s). The response to LPS lymph was significantly attenuated by naproxen (Fig. 6). Addition of PGE2 (5 μM) to the naproxen
containing Krebs augmented baseline firing to 19.3 ± 2.8 (imp/s) and also fully restored the response to LPS lymph (Fig. 6). The mesenteric afferent response to LPS was attenuated by naproxen but this failed to reach significance (Fig. 7). In contrast, PGE2 in the presence of naproxen augmented the LPS-induced response (Fig. 7). LPS-mediated inhibition of spontaneous contractile activity persisted after treatment with naproxen but the duration of this inhibition was significantly reduced (169.0 ± 22.9 vs. 484.4 ± 78.4 s, P < 0.05).

The response to both LPS and LPS lymph was not attenuated by treatment with AH6809 (Fig. 8), and indeed the response to LPS was significantly augmented in animals treated with AH6809.

**DISCUSSION**

The present study provides new insight into the local effects of LPS on mesenteric afferent discharge. Mesenteric lymph containing mediators released from the gut during endotoxemia evoked a significant mesenteric afferent nerve discharge compared with lymph collected from control animals. This observation supports the concept that LPS-induced inflammation leads to increased mesenteric afferent discharge which may contribute to visceral hypersensitivity. Such gut-derived inflammatory products play a major role in shock-induced pathological responses (1, 11). Moreover, systemic LPS induces a wide range of effects including fever, sickness behavior, and hyperalgesia that depend in part on activation of afferents whose terminals lie within the bowel wall (15). Our hypothesis that inflammatory mediators within mesenteric lymph can participate in this process of hyperalgesia is supported by our present results. In some experiments control lymph also increases mesenteric afferent discharge. However, it should be borne in mind that even these control animals had undergone abdominal surgery to place the lymphatic cannula and may have developed a macrophage-mediated postoperative ileus (17). We attempted to minimize such a contribution by allowing 24-h recovery following surgery before collecting the lymph.
Another important observation in our study was that LPS itself also activated afferent discharge with a similar time course to the response seen with LPS lymph. This might suggest that LPS acts directly on the afferent endings or on other elements within the bowel wall to release mediators that act locally to augment afferent firing. The short latency of this response might imply an action on the LPS receptor that is present on macrophage and other structures within the bowel wall (2). This sensitivity to LPS raises the possibility that the afferent response to LPS lymph arises as a consequence of LPS itself entering the lymph following intraperitoneal administration and exerting a direct effect on primary afferents. Although we did not measure the concentration of LPS in the mesenteric lymph, this explanation would seem unlikely because the response to LPS lymph was markedly attenuated by pretreatment with naproxen, whereas LPS itself still could mediate a marked and robust response following treatment with the cyclooxygenase inhibitor naproxen.

The short latency for activation of mesenteric afferent discharge following the LPS lymph and LPS is remarkable, suggesting that mediators released following the LPS accumulate rapidly in the interstitial fluid within the gut wall. The rapid recovery of discharge following treatment would also imply that these mediators are rapidly eliminated following their release either by reuptake or dissipation through the rapid perfusion system. This time course is very different from that seen following LPS administration in vivo in which afferent discharge rises substantially 15 min after administration, coinciding with an augmenting sensitivity to both mechanical stimulation (distension) and chemical mediators (5-HT) (20). However, even in these in vivo experiments, a small transient increase in discharge was observed immediately following administration of LPS, and this may coincide with the actions seen in the present study in vitro (20).

LPS-induced inflammatory responses depend on the release of proinflammatory cytokines such as IL-1, IL-6, and TNF-α. Indeed, inflammatory cytokines acting locally may directly or indirectly stimulate and/or sensitize nociceptors, thereby contributing to sensory hyperalgesia (5, 16). Previous work implicating TNF-α in the development of neuropathic pain follows the observation that exogenous TNF-α elicits a PKA-dependent response in rat sensory neurons (29). However, in the present study, TNF-α at concentrations considerably higher than that found in mesenteric lymph after LPS treatment did not stimulate mesenteric afferent discharge. It appears, therefore, that the afferent response to LPS lymph was not induced by TNF-α alone. However, this does not rule out the possibility for TNF-α acting as part of a cascade of cytokine contributing to more longer-term changes in afferent sensitivity rather than the direct short-term effects that were observed in the present study (4).

Prostaglandins play an important role in communication between the gastrointestinal immune system and the enteric nervous system. During intestinal inflammation, prostaglandins are released from a variety of cell types, including sympathetic nerve terminals and immunocompetent cells (4, 26). It is well known that prostaglandins increase the sensitivity of sensory nerves to inflammatory mediators by acting on prostaglandin EP receptors expressed on both vagal and spinal afferent endings (13). The present study demonstrates that inflammatory mediators released from gut into lymph influence mesenteric afferent firing via a COX-related mechanism, since the response to LPS lymph was inhibited by the COX inhibitor naproxen. This observation is in agreement with the concept that prostanoids play a pivotal role in modulating visceral sensitivity during inflammation.

Fig. 7. Histogram showing the peak increase in afferent discharge in response to LPS lymph (top) and LPS (bottom). Naproxen significantly attenuated only the response to LPS lymph. In contrast, PGE₂ significantly augmented the response to both LPS lymph and LPS. For the former, this was sufficient to restore the LPS lymph response to a comparable level to that obtained in the absence of naproxen while the response to LPS in the presence of naproxen and PGE₂ significantly exceeded that to LPS alone.

Fig. 8. Histogram showing the response to LPS lymph (top) and LPS (bottom) in the absence and presence of the EP receptor antagonist AH6809. Note that far from attenuating these responses the peak discharge was higher after treatment, which in the case of LPS was significantly above the control response. P < 0.05.
There are at least two isoforms of the COX enzyme, COX-1 and COX-2. COX-1 is constitutively expressed and may be involved in controlling baseline visceral afferent sensitivity. However, during inflammatory conditions such as colitis, up-regulation of the COX-2 occurs, leading to augmented prostanoid synthesis, and this enzyme may therefore be important in hyperalgesia during inflammation (18). Naproxen inhibits both COX-1 and COX-2, but the short latency in duration of the responses observed in the present study would suggest that it is the constitutive isoform that is being influenced by naproxen in the present study. The background of prostanoids may serve to sensitize the afferent endings to other mediators (possibly also prostanoids) that are present in the LPS lymph. This interpretation is supported by the observation that the addition of PGE$_2$ to the bathing medium can restore the afferent response to LPS lymph.

PGE$_2$ evokes a powerful activation of mesenteric afferents and mediates the sensitivity of afferent responses to both IL-1$\beta$ and bradykinin (16, 23). In the gastrointestinal tract, EP$_1$ receptors appear to play a major role in direct activation of mucosal mesenteric afferents, while EP$_2$ receptors may also play a sensitizing role (13, 23). It is interesting that in the present study, the EP$_1$/EP$_2$ receptor antagonist AH6809 failed to mitigate the mesenteric afferent response to LPS lymph despite the marked influence of PGE$_2$ on the afferent response to LPS lymph. This may suggest that other subtypes of EP receptors, including EP$_3$ and EP$_4$, play a role in the response. On the other hand, PGE$_2$ is not the only prostanoid released by LPS that potentially could contribute to modulation of afferent sensitivity in this model.

Disturbances in intestinal motility following LPS was widely reported using both in vivo and in vitro models (6, 14, 22, 24, 25). Most studies reported inhibition of intestinal motility with intestinal motor dysfunction involving endogenous nitric oxide, prostanoids, and cytokines. Observations in the present study that jejunal contractile activity is inhibited by LPS and that this period of inhibition is decreased after naproxen is consistent with this view. The effect of LPS lymph on jejunal motility was less than that of LPS itself, because application of LPS lymph inhibited spontaneous contractions in only three of eight experiments, and even for these three, the duration of inhibition was much shorter than for LPS. The prolonged effect of LPS might be explained by an inflammatory cascade with multiple intermediate mediators, several of which inhibit motility. In contrast, LPS lymph, because it is likely to be made up of the end products of such a cascade, would be expected to have a shorter duration of action. Alternatively, initially active inflammatory mediators may have been catabolized or otherwise degraded by the time the LPS lymph was collected, thus providing another possible basis for the disparity in duration of action between LPS and LPS lymph.

In summary, the present study indicates that gut-derived mediators released by LPS have a profound effect on mesenteric afferent sensitivity. TNF-$\alpha$ is unlikely to have a direct influence on afferent excitability but prostaglandins appear to play a modulating influence.

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