Inducible and neuronal nitric oxide synthase involvement in lipopolysaccharide-induced sphincteric dysfunction

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Fan, Ya-Ping, Sushanta Chakder, Feng Gao, and Satish Rattan. Inducible and neuronal nitric oxide synthase involvement in lipopolysaccharide-induced sphincteric dysfunction. Am J Physiol Gastrointest Liver Physiol 280: G32–G42, 2001.—We examined the effect of endotoxin lipopolysaccharide (LPS) on the basal tone and on the effects of different stimuli and agonists and transcriptional and translational expression of nitric oxide (NO) synthase (NOS) isozymes in the lower esophageal sphincter (LES), pyloric sphincter (PS), and internal anal sphincter (IAS). NO release was also examined before and after LPS. LPS caused a dose-dependent fall in the basal tone and augmentation of the relaxation caused by nonadrenergic, noncholinergic (NANC) nerve stimulation in the LES and IAS. In the PS, LPS had no significant effect on the basal tone and caused an attenuation of the NANC relaxation and an augmentation of the contractile response of muscarinic agonist. Interestingly, the smooth muscle relaxation by atrial natriuretic factor was suppressed in the LES and IAS but not in the PS. These changes in the sphincteric function following LPS may be associated with increase in the inducible NOS (iNOS) expression since they were blocked by iNOS inhibitor l-canavanine. Augmentation of NANC relaxation in the LES and IAS smooth muscle by LPS may be due to the increased activity of neuronal NOS and NO production.

lower esophageal sphincter; pyloric sphincter; internal anal sphincter; nonadrenergic, noncholinergic nerve stimulation; vasoactive intestinal polypeptide; atrial natriuretic factor

ENDOTOXEMIA FOLLOWING infection with Gram-negative bacteria such as *Escherichia coli* is commonly associated with a number of serious clinical consequences, including gastrointestinal abnormalities such as vomiting, ileus, and diarrhea. The gastrointestinal abnormalities are broadly characterized by a decrease in gastric emptying (11, 32, 33, 35) and an increase in small intestinal transit (12, 34, 35). The endotoxin in some cases may also cause a decrease in the large intestinal transit (11). The endotoxin responsible for the motor disturbances in response to Gram-negative bacterial infection is lipopolysaccharide (LPS). The mechanism for the opposing effects of endotoxemia in the gastrointestinal tract is not known.

In contrast to the above-mentioned effects of the endotoxin on intestinal motility, its effects on the gastrointestinal tract in terms of inflammation and severe damage to the mucosa that disrupts the absorptive and secretory functions are well known. Although these actions may provide an explanation for the observed symptoms in the gastrointestinal tract, specific studies designed to examine the mechanism of these opposing effects on gastrointestinal motility in different parts of the gut have not been carried out. Moreover, the actions and mechanisms of action of the endotoxin in the sphincteric smooth muscle are not known.

Gastrointestinal sphincters are known to play a significant role in the regulation of gastrointestinal transit. Among other factors, the pyloric sphincter (PS) may be one of the regulators of gastric emptying (21). The internal anal sphincter (IAS), on the other hand, plays a major role in the regulation of continence and evacuation of the large intestinal contents (16, 29). At the present time, there are no specific data that examine the actions of LPS on any of the gastrointestinal sphincters.

The purpose of present investigation was therefore to examine the actions of LPS on the basal sphincteric tone and relaxation caused by nonadrenergic, noncholinergic (NANC) nerve stimulation and different agonists. The studies were also designed to examine the expression of different nitric oxide (NO) synthase (NOS) isozymes and to measure the release of NO in the muscle bath perfusate following the NANC nerve stimulation by electric field stimulation (EFS). These parameters were examined in controls and after LPS pretreatment. The studies specifically examined the role of inducible NOS (iNOS) and neuronal NOS (nNOS) in the observed changes in sphincteric function.

MATERIALS AND METHODS

Preparation of Smooth Muscle Strips

The smooth muscle strips from the lower esophageal sphincter (LES), PS, and IAS from opossums were prepared for the recording of isometric tension as described previously.

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ephrine were considered to be Emax, and all the contractile response to bethanechol and IAS tone in response to phenylephrine were determined for the stimulation of the respective sphincteric smooth muscle. The above-mentioned parameters consisted of a pair of platinum wires fixed at both sides of the smooth muscle strips. The electrodes used for the EFS were used. The optimal length and the baseline of the smooth muscle strips that developed spontaneous steady tension and relaxed in response to EFS were used. The optimal length and the baseline of the smooth muscle strips were determined as described previously (25, 27).

Measurement of Isometric Tension

The smooth muscle strips were secured at both ends with silk sutures and transferred to 2-ml muscle baths containing oxygenated Krebs solution (37°C). One end of the muscle strip was anchored at the bottom of the muscle bath, and the other end was attached to a force transducer (model PTO3; Grass Instruments, Quincy, MA) for the measurement of isometric tension on a Dynograph recorder (model R411; Beckman Instruments, Schiller Park, IL). The muscle strips were stretched initially at 10 mN of tension and then allowed to equilibrate for at least 1 h with regular washings at 20-min intervals. Only the smooth muscle strips that developed spontaneous steady tension and relaxed in response to EFS were used. The optimal length and the baseline of the smooth muscle strips were determined as described previously (25). At the conclusion of the experiments, all the tissues were cut below the suture material, blotted dry, and accurately weighed. The force of all the smooth muscle tissues was expressed in millinewtons. For the quantification of the fall in basal sphincteric tension, the smooth muscle relaxation in response to 5 mM EGTA at the end of the experiment in each smooth muscle was considered to be 100% (Emax), and all the relaxant responses were in reference to this. Likewise, the rise in the LES and PS basal tone in response to bethanechol and IAS tone in response to phenylephrine were considered to be Emax, and all the contractile responses were represented in millinewtons (23).

NANC Nerve Stimulation With EFS

EFS was delivered from a Grass stimulator (model S88) connected in series to a Med-Lab Stimu-Splitter II (Med-Lab Instruments, Loveland, CO). The Stimu-Splitter was used to amplify and measure the stimulus intensity using the optimal stimulus parameters for the neural stimulation (0.5-ms pulse duration, 200-400 mA, 4-s train) at varying frequencies of 0.5-20 Hz. For the quantitative analysis of LPS pretreatment on the NANC nerve-mediated relaxation of different sphincters, the appropriate voltages (12-15 V) were determined for the stimulation of the respective sphincteric smooth muscle strips. The electrodes used for the EFS consisted of a pair of platinum wires fixed at both sides of the smooth muscle strip. The above-mentioned parameters of EFS are known to selectively cause relaxation of the sphincteric smooth muscle via the activation of NANC myenteric neurons.

**Drug Responses**

Pretreatment with different doses of LPS (0.1–1 mg/kg im, 10 h before the study) was used to examine the effects on the basal sphincteric tone and response to NANC nerve stimulation and different agonists. All experiments (except for bethanechol and phenylephrine effects) were carried out in the presence of guanethidine (3 × 10⁻⁶ M) and atropine (1 × 10⁻⁶ M) where applicable. All the agonists were given in a cumulative fashion. Once the concentration-response curve to an agent was determined, the smooth muscle strips were washed at least six times, and the resting tension was allowed to recover to the preinjection levels.

The agonists that cause relaxation or contraction of the smooth muscle via specific pathways were used in all the LES, PS, and IAS tissues. Vasoactive intestinal polypeptide (VIP) and isoproterenol are known to activate adenylate cyclase (AC) via the activation of specific cytoplasmic membrane receptors coupled to a specific G protein linked to AC (31). Atrial natriuretic factor (ANF), on the other hand, is known to cause smooth muscle relaxation via the activation of a specific membrane receptor linked directly to guanylate cyclase (GC) (28). NO is considered to be primarily responsible for the NANC nerve-mediated relaxation of the smooth muscle via the activation of the intracellular soluble fraction of GC (8, 9, 22, 26). In the present studies, sodium nitroprusside (SNP) was used as an NO donor. Bethanechol was used as the contractile agonist in the LES and PS, and phenylephrine was used in the case of IAS smooth muscles. The use of different contractile agonists for different sphincters was a departure from the standard protocol that calls for the use of the same agonist in all sphincters. The reason for this was that bethanechol in the IAS was found to cause only a modest contraction of the smooth muscle. Likewise, phenylephrine caused a limited contraction of the LES and PS smooth muscles.

Detailed studies using L-canavanine (iNOS inhibitor) (13, 17, 18) to examine the involvement of iNOS following LPS were carried out by giving LPS and the iNOS inhibitor simultaneously. The doses of L-canavanine used have previously been shown to be selective in inhibiting iNOS (5, 17, 18). The majority of data were grouped into control (where animals received no treatment), LPS (LPS only), and LPS + L-canavanine.

To understand the role of different NOS isozymes in the action of LPS pretreatment, we performed Western and Northern blot analyses. To examine the influence of LPS on NANC nerve stimulation-induced sphincteric smooth muscle relaxation, NO release in the muscle baths was monitored in control vs. LPS experiments.

**Western Blot Studies**

Western blot analysis of iNOS, nNOS, and endothelial NOS (eNOS) in different gastrointestinal sphincteric smooth muscles of opossums in the absence or presence of LPS pretreatment was performed according to the protocols of Transduction Laboratories and Amersham Life Sciences. Briefly, the LES, PS, and IAS tissues were cut into small pieces and rapidly homogenized in 5 volumes of boiling lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, 10 mM Tris, pH 7.4) and then put into a microwave for 10 s. The homogenates were centrifuged (16,000 g at 4°C) for 15 min to pellet insoluble material, and the protein contents were determined by the method of Lowry et al. (19) using BSA as the standard. All of the samples were mixed with 2× sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol) and boiled for 4 min. Fifteen to
twenty microliters (20 μg total protein) of each sphincteric tissue sample was applied to 10% SDS-PAGE, and the separated proteins were electrophoretically transferred to nitrocellulose membrane at 4°C. To block nonspecific antibody binding, the nitrocellulose membranes were immersed overnight at 4°C in Tris-buffered saline-Tween (TBS-T, composition: 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) containing 5% nonfat dry milk. The nitrocellulose membranes were incubated with the diluted primary NO antibodies (iNOS 1:5,000; nNOS 1:500; eNOS 1:1,000) for 1 h at room temperature. After washing with TBS-T, the membranes were incubated with the horseradish peroxidase labeled-secondary antibody (donkey anti-rabbit IgG, 1:1,500) for 1 h at room temperature. The bands were identified by chemiluminescence using the ECL detection system and Hyperfilm MP (Amersham Life Science). The bands of three NOS types on the Hyperfilm were recorded with a scanner (SNAP SCAN.310; Agfa, Ridgefield Park, NJ), and the relative densities were calculated by using the NIH Image software program.

**Northern Blot Studies**

**Isolation of total RNA.** This was done in the following steps: tissue homogenization, RNA extraction, RNA precipitation, RNA wash, and solubilization. Briefly, the total RNA from the sphincteric smooth muscle tissues was isolated using the improvised method of Chomczynski and Sacchi (10) using TRI Reagent, following the instructions provided by the supplier of the reagent (Molecular Research Center, Cincinnati, OH). Briefly, the tissues were transferred to sterile polypropylene tubes and homogenized in TRI Reagent (1 ml/50–100 mg tissue) with a tissue homogenizer (Tekmar Tissuemizer; Tekmar, Cincinnati, OH) and the homogenate was stored at room temperature for 5 min. The homogenate was then supplemented with 0.2 ml chloroform/ml of the TRI Reagent, covered tightly, and shaken vigorously for 15 s. The homogenate was then left undisturbed for 10 min at room temperature and centrifuged at 12,000 g for 8 min. The aqueous phase was transferred to the fresh tube. The latter was mixed with isopropanol 0.5 ml/ml TRI Reagent and kept at room temperature for 10 min, and total RNA was isolated by centrifugation (12,000 g at 4°C for 8 min). The total RNA was then washed with 75% ethanol and centrifuged for 5 min at 7,500 g at 4°C. The RNA pellet was air dried for 5–10 min and dissolved in FORMAzol (Molecular Research Center), and the samples were incubated at 55–60°C for 10 min. The concentration of the RNA was determined by measuring the absorbance at 260 nm.

**Preparation of iNOS cDNA probes.** Mouse macrophage iNOS cDNA was purchased from Alexis Biochemicals (San Diego, CA). The cDNA was radiolabeled (1 × 10⁶ cpm/μg) using [α-³²P]dCTP and the RadPrime DNA labeling system ( Gibco BRL, Gaithersburg, MD). The LES, PS, and IAS smooth muscles obtained from control and LPS-treated animals were used for the purpose.

**NO Determination**

Measurement of total NOx (NO + NO₂ + NO₃) was used to determine the release of NO by the tissues. Once released, NO is rapidly (within a few seconds) oxidized to NO₂ > NO₃. Therefore, for the accurate determination of NO release by the tissues, it is necessary to measure all forms of NO (6). Total NOx was determined by the NO chemiluminescence analyzer (model 270B; Sievers Instruments, Boulder, CO) specifically designed for this purpose. The method is a modification of the method originally used by Braman and Hendrix (4) and Bush et al. (6) and has been previously used in our laboratory (7).

The LES, PS, and IAS smooth muscles obtained from control and LPS-treated animals were used for the purpose. Muscle bath perfusates were collected under the following categories: basal (smooth muscles without any treatment), EFS (smooth muscles stimulated with 5 Hz of EFS), EFS-TTX (smooth muscles stimulated with EFS in the presence of TTX), and EFS-iNOS (smooth muscles stimulated with EFS in the presence of N-O-nitro-L-arginine (l-iNOS)). At appropriate times, the muscle bath perfusates (200 μl) were collected and rapidly stored in a 1.5-cm³ vial at −20°C. These samples were analyzed for NO determinations.

On each day of analysis, standard curves were generated after injection of 0, 1, 5, 10, 50, and 100 nmol of sodium nitrate (in 50-μl volume) into 300 ml of 0.1 M vanadium (III) chloride (Alrich, Milwaukee, WI) in a 5-ml test tube heated to 90°C in a dry bath incubator (Fisher Scientific, Pittsburgh, PA). Two minutes later, 100 μl of gas generated above the liquid was drawn and immediately injected into the septum on the top of the purge vessel of the NO analyzer. Contents of the vessel were constantly bubbled with oxygen-free nitrogen. The NOx signals from the chemiluminescence detector were displayed and analyzed by a PC-based data recording and analyzing system (Duo-18; World Precision Instruments, Sarasota, FL). The NOx values with the standards and the test samples (100 μl) were highly reproducible. Background concentrations of NOx with different agents in Krebs solution were measured and subtracted from the respective muscle bath perfusates. The standards and the test samples were analyzed in duplicate.

**Drugs and Chemicals**

The following chemicals were used in the study: bethanecol chloride, indomethacin, isoproterenol hydrochloride, LPS (from E. coli, serotype 026:B6); L-canavanine (iNOS inhibitor), L-NNA (general NOS inhibitor), phenylephrine, and SNP (all Sigma Chemical, St. Louis, MO); VIP (Bachem Bioscience, Torrance, CA); ANF (Peninsula Laboratories, San Carlos, CA); and EDTA tetrasodium.
The vials and pipette tips were siliconized, and the muscle baths were treated with 2.5% BSA to reduce the binding of peptides to the surface of glass or plastic.

Data Analysis

Data are given as means ± SE of different experiments. The basal tone and changes in the sphincteric tension in response to any of the agonists and stimuli in control vs. LPS pretreatment were calculated as the force in millinewtons as described. The maximal fall (or the passive force) in the basal tension in each smooth muscle strip was determined at the end of the experiment by the addition of excess EGTA until there was no further fall in the basal force. All of the data with a fall in the basal tension with the agonists and stimuli was expressed with reference to $E_{\text{max}}$ of the fall in the presence of EGTA. Percent fall in the basal tension was calculated by the ratio of decrease in force by the stimuli to the basal tone. The basal tone was calculated by subtracting passive force from the observed tension (15).

Statistical significance of the differences between different groups was determined by Student’s $t$-test. Two-way ANOVA was used for the comparison of entire concentration or frequency response curve before and after the treatment.

RESULTS

Influence of LPS Pretreatment on the Basal Tone of LES, the PS, and IAS

LPS caused a dose-dependent fall in the basal tone of the LES and IAS. On the other hand, it had no significant effect on PS tone (Fig. 1). In these series of experiments, the basal LES tone in the smooth muscle strips from the untreated animals was 48.35 ± 3.97 mN, and following 0.1, 0.5, and 1 mg/kg LPS it was significantly and dose-dependently decreased to 34.17 ± 3.70, 30.48 ± 2.56, and 23.69 ± 4.05 mN, respectively ($P < 0.05; n = 6$). The basal tone in the pyloric smooth muscle strips, on the other hand, before and after different doses of LPS was 4.54 ± 0.42, 4.21 ± 0.53, 4.92 ± 1.06, and 3.58 ± 0.64 mN, respectively, and was not significantly different from controls ($P > 0.05; n = 6$). The basal IAS tone in these series of experiments in control was 28.96 mN, and following different doses of LPS the values were 25.97 ± 3.04, 24.14 ± 3.22, and 18.62 ± 2.83 mN, respectively. The values in the presence of 0.5 and 1 mg/kg LPS were significantly lower ($P < 0.05; n = 6$).

The levels of basal tone in the LES, PS, and IAS and their changes by different agonists were found to be reproducible in different sphincters. This allowed the comparison of changes in different sphincters before and after the LPS pretreatment in various experimental groups as stated in MATERIALS AND METHODS.

It is noteworthy that the PS in most of the animals exhibited a steady-state low-grade basal tone. In addition, however, in some animals, there were slow (0.7–1.5/min) and fast (2–3.5/min) (averaged over 1-h periods) rhythmic patterns of phasic activity over and above the basal tone.

Influence of LPS Pretreatment on the Expression of Different NOS Isozymes in Different Sphincteric Smooth Muscles

To determine changes in the translational expression of different NOS isozymes following LPS, Western blot studies were performed on the LES, PS, and IAS tissues isolated from the animals without LPS and with LPS treatment. It was of interest that among all the NOS isozymes examined, only significant increases in iNOS protein following LPS were found in the LES and IAS ($P < 0.05; n = 6$). On the other hand, LPS had no significant effect on the basal tone of the PS ($P > 0.05; n = 6$). Values are means ± SE.
were elevated after LPS pretreatment in the LES and IAS ($P < 0.01; n = 4$; Fig. 3).

**Influence of iNOS Inhibitor on LPS-Induced Fall in the Basal Tone of LES, PS, and IAS**

On the basis of information on the expression of NOS isozymes in our studies and from the literature, we examined the effect of iNOS inhibitor l-canavanine on LPS-induced changes in the basal tone. For these experiments, LPS (0.5 mg/kg im) and l-canavanine (100 mg/kg im) were given at the same time at two different injection sites. Results show that l-canavanine caused significant blockade of the adverse effect of LPS on the basal tone in the LES and IAS ($**P < 0.01; n = 4$) but caused a modest one in the PS ($*P < 0.05; n = 4$).
sphincter, where LPS had no significant effect on the basal tone to begin with, the combination of LPS and L-canavanine caused no further change in the tone.

Influence of LPS on the Sphincteric Smooth Muscle Relaxation by NANC Nerve Stimulation Caused by EFS

To examine the influence of LPS on the NANC nerve-mediated relaxation of the sphincteric smooth muscles, first we examined the effects of LPS (0.1 and 0.5 mg/kg). In the LES and IAS but not PS, LPS caused a significant augmentation of the sphincteric smooth muscle relaxation by NANC nerve stimulation (Fig. 5). The augmentation was most marked in the case of lower frequencies of EFS. In the LES, the basal smooth muscle tone in control animals was 48.4 ± 4.0 mN, which fell to 34.8 ± 2.2 mN following 0.5 Hz of EFS. The corresponding values following 0.1 and 0.5 mg/kg LPS were 34.2 ± 3.7 and 16.6 ± 3.3 and 30.5 ± 2.6 and 12.4 ± 1.8 mN, respectively. On the contrary, LPS in the PS caused an attenuation of the NANC relaxation. Both the augmentative and inhibitory effects were partly blocked when LPS was given in combination with L-canavanine (Fig. 5).

Influence of LPS on NO Release in the LES, PS, and IAS

To ascertain the direct role of NO and site of its production in response to NANC nerve stimulation by EFS before and after LPS, NO release was determined by measuring the amount of total NOx as explained in MATERIALS AND METHODS. The standard curves for NOx generated by the addition of sodium nitrate (from 1 to 100 nmol) were linear, with a coefficient of correlation of 0.993. In all of the tissues, EFS caused a significant increase in NO production. The increases in NO were further augmented by LPS pretreatment in the LES.
and IAS but not PS. In the LES, PS, and IAS, NO production in control experiments following EFS (5 Hz) was 10.0 ± 1.3, 17.1 ± 1.3, and 24.0 ± 1.1 nmol, respectively. These values in the LES and IAS smooth muscle tissues obtained from LPS-treated animals were significantly increased to 72.0 ± 10.3 and 79.0 ± 18.9 nmol, respectively (P < 0.05; n = 4; Fig. 6), and the attenuated responses were not reversed by L-canavanine. The dose-response curves with ANF in control vs. LPS pretreatment in pylorus were not significantly different (P > 0.05; n = 6).

Influence of LPS Pretreatment on the Sphincteric Smooth Muscle Relaxation by VIP, Isoproterenol, and SNP

In contrast to the effect of ANF, the fall in the basal tension of different sphincteric smooth muscles caused by VIP and SNP was not significantly modified by LPS pretreatment (Fig. 8). Likewise, the
effects of isoproterenol in different sphincters were also not modified by LPS pretreatment. The data suggest that the AC pathway associated with G protein coupling with the membrane receptor responsible for the sphincteric smooth muscle relaxation in response to certain agonists remained intact following the LPS pretreatment.

The data (with ANF vs. SNP) suggest that the GC activation associated with the membrane receptor is reduced in the LES and IAS but leaves the responses to soluble GC activation unmodified. It is well known that it is primarily the soluble GC activation that is involved in the smooth muscle relaxation by NO. The exact significance of the differential actions of LPS on the GC stimulation by ANF vs. NO donor SNP is not known. The data on the effect of LPS on the NANC relaxation by EFS vs. SNP (Fig. 8) suggest that the augmentation of the NANC relaxation may be due to increase in the production rather than the sensitivity of NO to GC.

**Influence of LPS Pretreatment on the Sphincteric Smooth Muscle Contraction by Bethanechol or Phenylephrine**

Bethanechol was used to elicit the smooth muscle contraction in the LES and PS, and phenylephrine was used in the IAS. The reason for the use of different contractile agonists in different sphincters is explained in MATERIALS AND METHODS. In the PS, LPS caused a significant augmentation of the contraction ($P < 0.05$; $n = 6$), whereas in the LES and IAS the endotoxin had no significant effect ($P > 0.05$; $n = 6$). The rise in the basal PS smooth muscle tone in response to $1 \times 10^{-4}$ M bethanechol in the tissues obtained from untreated animals was $8.01 \pm 1.12$ mN, and following $0.5$ mg/kg LPS treatment it was significantly increased to $22.28 \pm 3.06$ mN. Interestingly, the augmented increase in response to bethanechol was nullified when LPS and L-canavanine were given simultaneously so that the responses in control experiments vs. following

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**Fig. 8. Influence of LPS on sphincteric relaxation by vasoactive intestinal polypeptide (VIP) and sodium nitroprusside (SNP).** A: LES; B: PS; C: IAS. Data show that LPS had no significant effect on the overall dose-response curves of VIP and SNP in any of the sphincteric smooth muscles ($P > 0.05$; $n = 6$).
the combination were not significantly different ($P > 0.05$; $n = 6$; Fig. 9).

**DISCUSSION**

Previous studies have suggested that endotoxemia following bacterial infection or the endotoxin LPS leads to increase in the intestinal transit and decrease in gastric emptying. The mechanism for the diverse effects of the endotoxin on these gastrointestinal events is not known. The changes in gastrointestinal smooth muscle activity in the present study may be typified by those in the LES and IAS smooth muscles. In the LES and IAS, LPS caused a dose-dependent fall in the basal tone and augmentation of the NANC relaxation. Such changes in the gastrointestinal smooth muscle are believed to be responsible for the increased progression of intestinal contents. The changes in the PS, on the other hand, were different from those of LES and IAS and may partly contribute to the decrease in gastric emptying. The observed changes in the sphincteric smooth muscle tone appear to be associated with increases in iNOS expression. Augmentation of the NANC relaxation following LPS, on the other hand, may be associated with increased activity in nNOS leading to increase in NO release.

It is well known that the gastrointestinal sphincters play an important role in the regulation of gastrointestinal transit. However, none of the previous studies examined the effect of LPS on different gastrointestinal sphincters. The present studies were designed to examine the effect of LPS in different sphincters and to use them as models to determine the mechanism of diverse actions of LPS in gastrointestinal motility. In the LES and IAS, LPS caused a fall in the basal tone and augmentation of the NANC relaxation. In the PS, LPS had no significant effect on basal tone and caused an attenuation of NANC relaxation and augmentation of the smooth muscle contraction by muscarinic receptor activation.

The observed changes caused by LPS in the LES and IAS in the basal state and in response to different stimuli were tissue and stimulus specific. LPS-induced changes in the basal LES and IAS tone were absent in the PS. Furthermore, the responses to contractile agonists in the LES and IAS (bethanechol and phenylephrine in the case of LES and IAS, respectively), in contrast to PS, were not modified by LPS pretreatment. The fall in the basal tone of LES, PS, and IAS smooth muscles caused by the direct smooth muscle relaxants VIP, isoproterenol, and SNP were not modified by LPS pretreatment.

Systematic progression of intestinal contents is known to be critically dependent on the relaxation of the smooth muscle sphincters, descending inhibition, and ascending contraction (14, 24, 36). The observed changes in the LES and IAS (fall in the basal tone and augmentation of NANC relaxation) may explain a generalized mechanism following the endotoxin treatment to be responsible for the increases in the intestinal transit.

According to the present data, in relation to the PS, three factors may partly contribute to the decrease in the gastric emptying following LPS pretreatment. First, LPS causes an inhibition of the NANC relaxation in the pylorus. Second, LPS in the pylorus compared with the other sphincters was found to cause no significant change in the basal tone. Third, LPS caused an increase in response to the muscarinic receptor stimulation. An increase in response to muscarinic receptor stimulation by endogenously released acetylcholine from the vagal fibers either at the pre- or postganglionic level (1) may explain some of the changes in the PS following LPS. The inhibitory and augmentory actions of LPS may be partly associated with the stimulation of iNOS since they were blocked by L-canavanine. Such a suggestion is similar to the one made by the earlier studies (33). Whether this is related to an interaction between different NOS isozymes and increased musca-

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**Fig. 9.** Influence of LPS pretreatment on the rise in basal tone of the LES (A), PS (B), and IAS (C) caused by the contractile agonist bethanechol used in the LES and PS and by phenylephrine in the IAS. Note that LPS caused no significant effect on the contractile responses in the LES and IAS, whereas it caused a significant augmentation in the PS ($^{*}P < 0.05$; $n = 6$). The augmentation in PS was reversed by L-canavanine. Since LPS by itself had no significant effect on the contractile agonists in the LES and IAS, data with the influence of L-canavanine in these tissues are omitted.
rinic receptor activation is not known. Effects of LPS unrelated to PS that may also regulate gastric emptying (21) were not examined in the present study.

LPS-induced fall in the basal tone of the LES and IAS and augmentation of the NANC relaxation may be associated with increase in iNOS expression. First, LPS caused a selective increase in iNOS protein and mRNA in both the LES and IAS without significant changes in the expression of other NOS isoforms. Second, the observed changes in the basal tone and NANC relaxation in the LES and IAS were blocked by the simultaneous administration of the iNOS inhibitor L-canavanine and LPS. However, a direct role of associated changes in the iNOS expression in the observed functional changes in the sphincteric smooth muscles and the exact source of increased iNOS expression remain to be determined.

The mechanism of augmentation in the NANC relaxation in the LES and IAS may be discussed by considering the following possibilities: an increase in nNOS expression, an increased activity of nNOS leading to the increased release of NO, and increased sensitivity of NO at the smooth muscle cells. Since no increase in nNOS expression and in response to NO donor following LPS in either the LES or IAS was found, the remaining possibility was an increased release of NO by increased nNOS activation. This possibility is supported by the increased release of NO accompanying the LES and IAS smooth muscle relaxation by NANC nerve stimulation following LPS. Furthermore, the augmented release of NO was blocked by TTX and L-NNa. The effect of L-canavanine in NO release in these experiments was not investigated. On the contrary, in the PS, the attenuated NANC relaxation may be due to the downregulation of constitutive NOS (eNOS and nNOS) by the increased iNOS (3, 20, 30). However, no noticeable decrease in NO release following NANC nerve stimulation in the presence of LPS in the present studies was observed, suggesting a decrease in the sensitivity to NO. This was supported by attenuation of SNP responses in the PS in certain experiments.

An additional and interesting outcome of the present studies was that LPS pretreatment caused a distinct attenuation of ANF-induced fall in the LES and IAS smooth muscle tone that was not modified by L-canavanine. ANF-induced relaxation of the smooth muscle is known to be due to the activation of a membrane receptor that is directly linked to GC and is different from that of NO-related smooth muscle relaxation (22, 26–28). An increase in ANF following LPS treatment has been shown before (2), but there are no previous data that show changes in the sensitivity of ANF. Whether attenuation of responses to exogenous ANF in the LES and IAS is related to the downregulation of ANF receptors or desensitization to the elevated levels after LPS treatment and the significance of these findings remain to be elucidated.

In summary, the present studies suggest that an increase in intestinal transit may be partly explained on the basis of gastrointestinal smooth muscle inhibition (typified by the fall in the basal tone of the LES and IAS smooth muscles) and an increase in the NANC inhibitory neurotransmission. The fall in sphincteric smooth muscle tone may be associated with the increases in iNOS expression. Augmentation of NANC relaxation, on the other hand, involves an increase in nNOS activity, causing increased release of NO. The relative contribution of observed changes in different sphincteric smooth muscle function in LPS-induced changes in the gastrointestinal transit and delay in gastric emptying remains to be determined.

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