Upregulation of iNOS by COX-2 in muscularis resident macrophage of rat intestine stimulated with LPS

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Hori, Masatoshi, Muneto Kita, Shigeko Torihashi, Shigeki Miyamoto, Kyung-Jong Won, Kohichi Sato, Hiroshi Ozaki, and Hideaki Karaki. Upregulation of iNOS by COX-2 in the muscularis resident macrophage of rat intestine stimulated with LPS. Am J Physiol Gastrointest Liver Physiol 280: G930–G938, 2001.—We investigated the effect of lipopolysaccharide (LPS) on the induction of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in muscularis resident macrophages of rat intestine in situ. When the tissue was incubated with LPS for 4 h, mRNA levels of iNOS and COX-2 were increased. The majority of iNOS and COX-2 proteins appeared to be localized to the dense network of muscularis resident macrophages immunoreactive to ED2. LPS treatment also increased the production of nitric oxide (NO), PGE2, and PGI2. The increased expression of iNOS mRNA by LPS was suppressed by indomethacin but not by NG-monomethyl-L-arginine (L-NMMA). The increased expression of COX-2 mRNA by LPS was affected neither by indomethacin nor by L-NMMA. Muscle contractility stimulated by 3 μM carbachol was significantly inhibited in the LPS-treated muscle, which was restored by treatment of the tissue with L-NMMA, aminoguanidine, indomethacin, or NS-398. Together, these findings show that LPS increases iNOS expression and stimulates NO production in muscularis resident macrophages to inhibit smooth muscle contraction. LPS-induced iNOS gene expression may be mediated by autocrine regulation of PGs through the induction of COX-2 gene expression.

inducible nitric oxide synthase; intestinal motility; nitric oxide; prostaglandin

IT IS WELL KNOWN that lipopolysaccharide (LPS) or inflammatory cytokines such as interleukin (IL)-1 drive both cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) gene expression in macrophages, resulting in increases in the release of PGs and nitric oxide (NO) (2, 13, 18, 21, 24). Some reports indicate that there may be cross-talk between the COX-2 and iNOS genes in macrophages. For example, in the J744 macrophage cell line, PGE2, PGI2, and 8-bromoadenosine 3',5'-cyclic monophosphate suppress iNOS protein expression induced by LPS (31). Also, in LPS-stimulated J744 cells (27), low and high concentrations of PGE2 upregulate and downregulate iNOS expression, respectively. In the ANA-1 macrophage cell line, release of PGE2 induced by LPS was blocked by an iNOS inhibitor, aminoguanidine (32). In addition, in rat lung, COX-2 expression by acute hypoxia was suppressed by sodium nitroprusside and stimulated by dibutyryl cAMP (6). These reports suggest that although the cross-talk between iNOS and COX-2 exists in macrophage cells, their relationship is complex and cell type specific (see Ref. 15 for review).

Immunologically active cells, including macrophages in the intestinal mucosa, are thought to be closely related to the inflammatory bowel diseases. Recently, macrophages in the muscle layer were reported as a population distinct from mucosal macrophages in mammalian intestines (20, 25, 26). These macrophages are regularly distributed in the subserosa, at the level of the myenteric plexus, and inside the muscle layer. Although their unique distribution and great number imply an important role in the muscle layer, the function of those muscularis resident macrophages remains to be clarified and their pathological effects on the gastrointestinal tract have not yet been demonstrated. Recently, Schroeder et al. (34) and Eskandari et al. (11) reported that the macrophages induce NOS (iNOS) to release NO, which results in suppressed smooth muscle motility. These reports strongly suggest that the intestinal muscularis resident macrophage network should be an important mediator of endotoxin-induced gut dysmotility. However, the interaction between iNOS and COX-2 is not well investigated in the intestinal resident macrophages during endotoxemia.

The aim of the present study was to clarify the cross-talk between iNOS and COX-2 in the muscularis resident macrophages of rat small intestine stimulated by LPS. We found that LPS-induced iNOS gene expression might be mediated by autocrine regulation of PGs through the induction of COX-2 gene expression. The released NO plays an important role in mediating...
intestinal circular muscle contractility in the presence of endotoxin.

MATERIALS AND METHODS

Muscle preparation and measurement of muscle tension. Male Wistar rats were stunned and killed in accordance with the use and treatment of animals outlined in the Guide to Animal Use and Care of the University of Tokyo. The ileum from each rat was then dissected into 2- to 3-cm-long segments and cut open along the mesenteric attachment, and the mucosa and submucosa were removed. The remaining muscle layers were incubated with physiological salt solution (PSS) containing (in mM) 136.9 NaCl, 5.4 KCl, 1.0 MgCl₂, 23.8 NaHCO₃, 1.5 CaCl₂, and 5.5 glucose. PSS was aerated with 95% O₂-5% CO₂ to adjust pH to 7.3 at 37°C.

Muscle tension was recorded isometrically using a force-displacement transducer. Each muscle strip was attached to a holder in an organ bath (10 ml) containing PSS with a resting tension of 10 mN and equilibrated for 30 min to obtain a stable contractility induced by 72.7 mM KCl. The muscle strips were treated with PSS alone or with 100 μg/ml LPS dissolved in PSS in the organ bath for 4 h. Muscle tension was considered as the reference response (100%).

Quantitative RT-PCR analysis. Total RNA was extracted from the circular smooth muscle strips by the acid guanidinium isothiocyanate-phenol-chloroform method and the concentration of RNA was adjusted to 1 μg/μl with RNase-free distilled water. Quantitative RT-PCR was performed as follows. First-strand cDNA was synthesized using random 9-mer primer and avian myoblastosis virus (AMV) Reverse Transcriptase XL at 30°C for 10 min, 55°C for 30 min, 99°C for 5 min, and 4°C for 5 min. PCR amplification was performed by the hot starting method using Taq Gold (Perkin-Elmer, Branchburg, NJ). The oligonucleotide primers for iNOS designed from rat macrophage (37) were CTA CCT ACC TGG GGA ACA CCT GGG (forward) and GGA GGA GCT GAT GGA GTA GTA GCG G (reverse), and the suitable size of synthesized cDNA was 442 bp. The oligonucleotide primers for COX-2 designed from rat (12) were CTG TAT CCC GCC CTG CTG GTG (forward) and ACT TGC GTT GAT GTT GGC TGT CCT (reverse), and the suitable size of synthesized cDNA was 282 bp. The oligonucleotide primers for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (14) used were TAC CAG CCG GGG GAC CAC (forward) and CGA GCT GAC AGA GTA GTA (reverse) or CTG GCA TGG CCT TCG TGT TC (forward) and CCT GCT CTC TCA GTA TCC TTG CTG GGC T (reverse). The suitable sizes of the synthesized cDNA of GAPDH were 308 bp in the former primers and 366 bp in the latter primers. After initial denaturation at 95°C for 10 min, 24–40 cycles (4-cycle interval) of amplification at 94°C for 40 s, 55°C for 1.0 min, and 72°C for 1.5 min were performed using a thermal cycler (Takara PCR Thermal Cycler MP, Takara Biomedicals, Tokyo, Japan). PCR products in each cycle were electrophoresed on 2% agarose gel containing 0.1% ethidium bromide. The possible contamination of DNA was excluded by PCR with total RNA without the reverse transcription step. Detectable fluorescent bands were visualized by an ultraviolet transilluminator using FAS-III (Toyobo, Tokyo, Japan), and the area was measured using NIH Image software.

Immunohistochemistry. For immunohistochemistry, muscle strips were fixed with either 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for detecting iNOS or in Zamboni solution for detecting cyclooxygenase-1 (COX-1) and COX-2, and they were processed for whole mount preparations. Samples were incubated overnight at 4°C with anti-iNOS antibody (1:1,000; Transduction Labs, Lexington, KY), anti-rat resident macrophage antibody (9) (ED2, 1:500; Serotec, Oxford, UK), anti-COX-1 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-COX-2 antibody (1:50; Santa Cruz Biotechnology). They were then treated with an ABC kit (Vector, Burlingame, CA), and reaction products were detected by 0.03% diaminobenzidine and H₂O₂ in 0.05 M Tris–HCl buffer (pH 7.6). To check the specificity of the immunohistochemistry tests, tissues in which primary antibodies were omitted from the initial incubation were also prepared.

Muscle strips were also double-stained with the combination of anti-iNOS antibody and ED2 as well as with anti-COX-2 and ED2. iNOS and COX-2 or ED2 were detected with Texas red-conjugated streptavidin (1:100; Vector), or FITC-conjugated anti-mouse IgG (1:100; Sigma, St. Louis, MO), respectively. Colocalization was analyzed using a confocal laser scanning microscope (MRC-1024; Bio-Rad, Hercules, CA). iNOS and COX-2 could not be stained simultaneously because the fixing condition is different for each protein.

Measurement of released PGs. Each muscle strip was attached to a holder in an organ bath (2 ml) containing PSS and equilibrated for 30 min. The muscle strips were then treated with or without 100 μg/ml LPS for 4 h at 37°C. After incubation with PSS alone or PSS + LPS for 4 h, 50 μl of the solution were removed, and the released PGE₂ and PGI₂ were measured using an enzyme immunoassay system (Amersham Pharmacia Biotech, Tokyo, Japan). Released PGs were calculated using the standard assay in the kit and expressed as picograms per milligram of wet weight.

Measurement of released NO. Each muscle strip was treated with or without LPS under the same conditions as those for the measurements of PGs. After incubation of the muscle strips with PSS alone or PSS + LPS for 4 h, 50 μl of the solution were removed, and the released NOS-2 and PGI₂ were measured using an enzyme immunoassay system (Amersham Pharmacia Biotech, Tokyo, Japan). Released nitrite was measured as an indicator of released NO by means of colorimetric assay for determination of total nitrite using Greiss reagent (Bioxytech nitric oxide assay kit, Oxis International, Portland, OR). The released nitrite content was expressed as micromoles per milligram of wet weight.

Statistics. Numerical data are expressed as means ± SE. Differences between mean values were evaluated by Student's t-test, and, where appropriate, analysis of variance (one-way ANOVA; Bonferroni's test) was performed.

RESULTS

Effect of LPS on induction of iNOS and COX-2 mRNAs. RT-PCR analysis was performed on RNA extracted from tissue treated with or without LPS (100 μg/ml) for 4 h at 37°C in the organ bath. As shown in Fig. 1, expression of the RT-PCR product encoding GAPDH (308 or 366 bp) was identical in the control and LPS-treated tissues. In contrast, iNOS (442 bp) and COX-2 (282 bp) mRNAs were strongly expressed in LPS-treated tissues. Total nitrite was measured as an indicator of released NO by means of colorimetric assay for determination of total nitrite using Greiss reagent (Bioxytech nitric oxide assay kit, Oxis International, Portland, OR). The released nitrite content was expressed as micromoles per milligram of wet weight.
In muscle incubated with normal PSS for 4 h, in contrast, mRNAs of COX-2 or iNOS did not increase.

Immunohistochemistry of iNOS and COX-2. Because intestinal tissue, even after separation from the mucosal layer, is composed of multiple types of cells, we performed immunohistochemistry using anti-iNOS and anti-COX-2 antibody. In the whole-mount tissue preparation without LPS treatment, iNOS and COX-2 proteins were not detected in the muscle layer; however, in muscle treated with LPS for 4 h, cells expressing iNOS (Fig. 3A) or COX-2 (Fig. 3B) immunoreactivities were observed among the smooth muscle cell layer. iNOS- or COX-2-immunoreactive cells were apparently muscularis resident macrophages but not smooth muscle cells.

We next attempted to confirm the immunoreactive cells as macrophages using the anti-resident macrophage antibody ED2 (9). As shown in Fig. 4A, iNOS-immunopositive cells also showed immunoreactivity for ED2. In addition, COX-2-expressing cells were also immunoreactive for anti-macrophage antibody ED2 (Fig. 4B). In this study, we also examined the immunohistochemistry of COX-1. LPS treatment for 4 h did not change the immunoreactivity for anti-COX-1 antibody in the resident macrophages and smooth muscle cells (data not shown).

Effect of LPS on NO release. We next measured total nitrite content as an indicator of NO released from the intestinal muscles in the absence or presence of LPS for 4 h (Fig. 5A). In the absence of LPS, the total released nitrite content was 0.15 ± 0.15 μmol/mg wet wt (n = 4). After incubation of muscle strips with LPS for 4 h, the total nitrite content was significantly increased, as shown in Fig. 5A (2.16 ± 0.73 μmol/mg wet wt; *P < 0.01, n = 4).

Effect of LPS on PG release. Because LPS induces COX-2 mRNA and protein in myenteric resident macrophages, we next examined the amount of PGs released from the tissue using an enzyme immunoassay method (Fig. 5B). In muscle not stimulated by LPS, the amounts of PGE_{2} and PGI_{2} were 1.14 ± 0.32 (n = 4) and 2.53 ± 0.62 (n = 4) pg/mg wet wt, respectively. After incubation of muscle tissue with LPS for 4 h, released PGE_{2} and PGI_{2} were increased to 10.5 ± 2.64 (P < 0.01) and 6.22 ± 0.32 (P < 0.01) pg/mg wet wt, respectively.
Effects of indomethacin and L-NMMA on the expression of iNOS and COX-2 mRNAs. We next examined the effects of a COX inhibitor, indomethacin, and a nonselective NOS inhibitor, L-NMMA, on the expression of COX-2 and iNOS mRNAs. As shown in Fig. 6, COX-2 mRNA levels increased by LPS were not affected by treatment of the tissue with indomethacin (10 μM) or L-NMMA (300 μM). In contrast, the increased iNOS mRNA level induced by LPS was decreased by treatment with indomethacin but not by L-NMMA.

Effects of LPS on muscle contractions. Finally, we examined the effects of LPS on smooth muscle contractility. In control tissue, 3 μM of carbachol induced contractions, the amplitude of which was almost identical to that of the contractions induced by 72.7 mM KCl (93.5 ± 2.17% of high K⁺-induced contractions; n = 43). Treatment of the tissue with PSS alone for 4 h slightly inhibited the 3 μM carbachol-induced contractions (72.3 ± 3.35% of carbachol-induced contraction before treatment of PSS for 4 h; n = 9). Treatment of muscle strips with a nonselective NOS inhibitor, L-NMMA (300 μM), for 4 h did not reverse this contraction (78.1 ± 11.0% of carbachol-induced contraction before treatment of PSS for 4 h; n = 10). Treatment of
the tissue with 100 μg/ml LPS for 4 h inhibited the carbachol-induced contraction. Combined treatment with LPS and l-NMMA for 4 h restored the suppressed muscle force. Indomethacin (10 μM) also restored the attenuated carbachol-induced contraction to the level obtained in the muscle without LPS treatment. Treatment of muscle strips with LPS and aminoguanidine (300 μM), a selective iNOS inhibitor, or NS-398 (10 μM) restored the attenuated carbachol-induced contraction to the level obtained in the muscle without LPS treatment. Treatment of muscle strips with LPS and aminoguanidine (300 μM), a selective iNOS inhibitor, or NS-398 (10 μM) restored the carbachol-induced contraction to the level obtained in the muscle without LPS treatment.

Fig. 3. Immunohistochemistry of anti-iNOS antibody (A) and anti-COX-2 antibody (B). Before incubation with LPS, macrophages did not express iNOS or COX-2 immunoreactivities in the whole mount preparations shown in left panels. After 4-h incubation, cells positive for iNOS (A) or COX-2 (B) were increased in right panels. They were distributed evenly in the muscle layer. Scale bar, 100 μm. All panels show typical results from 3–4 experiments.

Fig. 4. Confocal micrographs of iNOS (A) or COX-2 (B)-immunopositive macrophages in tissue after 4-h incubation with LPS. A: left, iNOS-immunopositive cells indicated by Texas red; middle, macrophages stained with ED2 by FITC in the same area as on left; right, merged image of left and middle panels. iNOS-immunopositive cells (arrowheads) were stained with ED2 at the same time. B: left, COX-2-immunopositive cells indicated by Texas red; middle, macrophages stained with ED2 by FITC in the same area as on left; right, merged image of left and middle panels. COX-2-immunopositive cells (arrowheads) show immunoreactivity for ED2. Typical results are shown from 3–4 experiments. Scale bar, 50 μm.
m), a selective COX-2 inhibitor, for 4 h also suppressed the inhibitory effect of LPS. The effects of indomethacin, NS-398, l-NMMA, and aminoguanidine on LPS-induced inhibition of muscle force were not significantly different from each other.

**DISCUSSION**

Bacterial toxins, particularly endotoxins such as LPS, can activate macrophages to induce iNOS and COX-2 (16–19, 27, 30). In this study, exposure of ileal circular smooth muscle to LPS for 4 h increased mRNA levels of both iNOS and COX-2, as shown in Fig. 1. The majority of iNOS and COX-2 proteins appeared to be restricted in ED2-positive resident macrophages, based on the results of double staining of ED2 and iNOS or COX-2 (Figs. 3 and 4). Similar results in regard to iNOS expression were obtained in our previous work (35). These results indicate that induction of iNOS gene and COX-2 gene was mainly exhibited in muscularis resident macrophages but not in intestinal smooth muscle cells. However, we cannot rule out the possibility of weak induction of these genes in smooth muscle cells, because it is difficult to compare the expression in the smooth muscle layer quantitatively. We also cannot completely rule out the contribution of a minor component of immunological cells such as mast cells, T cells, and natural killer cells within the rat intestinal muscle layer (20). In addition, a minor population of ED2-positive cells expressed neither iNOS nor COX-2, as shown in Fig. 4, indicating the possibility of heterogeneity in the muscularis resident macrophage. Further experiments are necessary to clarify this point.

Consistent with COX-2 mRNA induction and protein expression, incremental releases of PGE2 and PGI2 from the tissue were observed after LPS treatment. We also confirmed the increment of NO in the medium treated with LPS, as an indicator of NO, as shown in Fig. 5A.

It has been reported that expression of iNOS is stimulated by cAMP elevation in many cell types. In
macrophages, however, the effect of increased cAMP on iNOS expression is variable (15). Therefore, we next examined the interaction between iNOS and COX-2 in muscularis resident macrophages in situ. In the presence of a nonselective COX inhibitor, indomethacin (10 μM), the induction of iNOS mRNA by LPS was completely inhibited whereas the induction of COX-2 mRNA was unaffected. On the other hand, neither iNOS mRNA nor COX-2 mRNA expression was affected by a nonselective NOS inhibitor, L-NMMA. These results suggest that expression of COX-2 may be essential for LPS-induced iNOS gene expression in muscularis resident macrophages. In the time courses of mRNA expression in iNOS and COX-2, the increment of COX-2 mRNA expression was faster than that of iNOS mRNA expression, supporting the suggestion.

In agreement with these results, Milano et al. (27) reported that the expression of iNOS is stimulated by exogenously applied PGE₂ in the J774 murine macrophage cell line. In addition, most recently it was reported that LPS increased cAMP level via COX-2 induction and PGE₂ production, resulting in iNOS expression to produce NO in RAW264.7 macrophages (5).

Also working with a murine macrophage cell line, ANA-1, Perkins and Kniss (32) reported that NO is necessary for maintaining prolonged COX-2 gene expression. In the present study, however, the iNOS and COX-2 mRNA levels stimulated by LPS were not affected by the inhibition of NO production with L-NMMA, suggesting that, in the intestinal resident macrophage, NO does not regulate the iNOS and COX-2 genes.

We also observed that after treatment of intestinal tissue with LPS for 4 h in vitro, carbachol-induced contraction was significantly inhibited. The reduced contractility was restored by L-NMMA (Fig. 7) without changing the iNOS mRNA level (Fig. 6), suggesting that increased production of NO is responsible for the reduced smooth muscle contractility. A selective iNOS inhibitor, aminoguanidine, also suppressed the inhibitory effect of LPS on carbachol-induced contractions. In addition, we showed the increment of released NO in muscle treated with LPS for 4 h (Fig. 5A).

Interestingly, the contractility reduced by LPS was also restored by indomethacin. These results support the finding that indomethacin inhibited the LPS-induced increase in iNOS mRNA, as shown in Fig. 6. We further demonstrated that a selective COX-2 inhibitor, NS-398 (10 μM), also restored the LPS-induced inhibitory effects of muscle force (Fig. 7) and that PGE₂ and PGI₂ were released in LPS-treated muscles (Fig. 5B). In addition, immunohistochemical analysis indicated that the level of COX-1 protein expression did not change after LPS treatment. These results suggest that the elevated production of PGE₂ and PGI₂ may be attributable to COX-2 but not to COX-1.

As for the molecular mechanism by which PGs up-regulate iNOS induction, it has been reported that the transcription of iNOS genes in macrophages is regulated mainly by the nuclear factor (NF)-κB transcription family (22, 38). The promoter of the murine gene encoding iNOS contains two κB binding sites (23), and protein binding to the κB binding sites is necessary to confer inducibility by LPS (38). Thus PGs produced by LPS treatment may bind to the EP3 receptor to stimulate the adenylate cyclase/cAMP pathway in the resident macrophage. In rat intestinal resident leukocytes, ED9- and CD14 (specific antibody for LPS receptor)-positive leukocytes make up ~55% of total leukocytes (5), suggesting that these ED9-positive cells, which may be ED2-positive resident macrophages, should be target cells of LPS in the smooth muscle layer. As is reported in cultured macrophage cell lines (28, 29), activated cAMP-dependent protein kinase (PKA) phosphorylates NF-κB inhibitory protein (1κB) to induce the activation of NF-κB. A pathway that is independent of the upregulation of NF-κB has also been reported (16, 17). Further studies are required to clarify the molecular mechanism of iNOS expression in the muscularis resident macrophage. However, most recently, Chen and co-workers (5) demonstrated that LPS increases cAMP via COX-2 induction and PGE₂ production, resulting in PKA activation to induce iNOS expression and NO production via NF-κB activation in the RAW264.7 macrophage cell line. This report strongly supports our conclusions in muscularis resident macrophages.

In the present study, we found that LPS stimulates muscularis resident macrophages to produce COX-2 and iNOS, resulting in inhibited muscle motility in rat ileum. However, in human mononuclear phagocytes, the ability to generate NO stimulated by LPS is lower than in mouse and rat macrophages, indicating the possibility that human macrophages cannot generate enough NO (8, 36). In contrast, a number of recent
studies documented that the capability for iNOS activity can be induced in some human macrophage cell lines (33) or in the presence of unconventional stimuli such as infection with human immunodeficiency virus type 1 (4). These reports indicate that iNOS expression may depend on the state of macrophage differentiation, the complex of stimuli, or tissue location. In addition, LPS alone has been shown to stimulate COX-2 induction in several cell lines of human macrophages (1, 3, 10). These results suggest that LPS-stimulated muscularis resident macrophages may play a critical role in the pathogenesis of gastrointestinal dysfunction during sepsis and multisystem organ failure in humans.

In summary, LPS induces iNOS gene expression in the muscularis resident macrophage in rat small intestine and releases NO to inhibit smooth muscle contractility. The effect of LPS seems to be mediated by the upregulation of COX-2 followed by the production of PGs that stimulates iNOS expression in an autocrine manner. These findings implicate the role of iNOS and COX-2 in the muscularis resident macrophage network in the pathogenesis of gastrointestinal dysfunction during sepsis and multisystem organ failure.

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