EP2 and EP4 receptors on muscularis resident macrophages mediate LPS-induced intestinal dysmotility via iNOS upregulation through cAMP/ERK signals

Tsuyoshi Tajima,1,2 Takahisa Murata,1 Kosuke Aritake,3 Yoshihiro Urade,3 Masaki Michishita,4 Toshiyuki Matsuoka,5 Shuh Narumiya,5 Hiroshi Ozaki,1 and Masatoshi Hori1

1Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, and 2Laboratory of Veterinary Pharmacology, Nippon Veterinary and Life Science University, Tokyo; 3Department of Molecular Behavioral Biology, Osaka Bioscience Institute, Osaka; 4Laboratory of Veterinary Pathology, Nippon Veterinary and Life Science University, Tokyo; and 5Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Submitted 7 July 2011; accepted in final form 4 December 2011

Tajima T, Murata T, Aritake K, Urade Y, Michishita M, Matsuoka T, Narumiya S, Ozaki H, Hori M. EP2 and EP4 receptors on muscularis resident macrophages mediate LPS-induced intestinal dysmotility via iNOS upregulation through cAMP/ERK signals. Am J Physiol Gastrointest Liver Physiol 302: G524–G534, 2012. First published December 8, 2011; doi:10.1152/ajpgi.00264.2011.—Intestinal resident macrophages play an important role in gastrointestinal dysmotility by producing prostaglandins (PGs) and nitric oxide (NO) in inflammatory conditions. The causal correlation between PGs and NO in gastrointestinal inflammation has not been elucidated. In this study, we examined the possible role of PGE2 in the LPS-inducible inducible NO synthase (iNOS) gene expression in murine distal ileal tissue and macrophages. Treatment of ileal tissue with LPS increased the iNOS and cyclooxygenase (COX)-2 gene expression, which lead to intestinal dysmotility. However, LPS did not induce the expression of iNOS and COX-2 in tissue from macrophage colony-stimulating factor-deficient op/op mice, indicating that these genes are expressed in intestinal resident macrophages. iNOS and COX-2 protein were also expressed in dextran-phagocytosed macrophages in the muscle layer. CAY10404, a COX-2 inhibitor, diminished LPS-dependent iNOS gene upregulation in wild-type mouse ileal tissue and also in RAW264.7 macrophages, indicating that PGs upregulate iNOS gene expression. EP2 and EP4 agonists upregulated iNOS gene expression in ileal tissue and isolated resident macrophages. iNOS mRNA induction mediated by LPS was decreased in the ileum isolated from EP2 or EP4 knockout mice. In addition, LPS failed to decrease the motility of EP2 or EP4 knockout mice ileum. EP2 or EP4-mediated iNOS expression was attenuated by KT-5720, a PKA inhibitor and PD-98059, an ERK inhibitor. Forskolin or dibutyryl-cAMP mimics upregulation of iNOS gene expression in macrophages. In conclusion, COX-2-derived PGE2 induces iNOS expression through cAMP/ERK pathways by activating EP2 and EP4 receptors in muscularis macrophages. NO produced in muscularis macrophages induces dysmotility during gastrointestinal inflammation.

Inflammation; intestinal motility; nitric oxide; prostaglandins

Inflammatory cytokines, such as interleukins and tumor necrosis factor-α, and chemical inflammatory mediators, such as bacterial wall components, prostaglandins (PGs), and nitric oxide (NO), induce gastrointestinal inflammation. Lipopolysaccharide (LPS) from gram-negative bacteria is a major causative factor of gastrointestinal inflammation (5). LPS stimulation activates nuclear factor κB (NF-κB) via a toll-like receptor-4 (TLR-4)-mediated signaling cascade that induces inflammatory-related substances, such as tumor necrosis factor-α, interleukin-1β, monocyte chemotactic protein-1, PGs, and inducible NO synthase (iNOS) (19, 22). Monocytes/macrophages are one of the most LPS-sensitive types of inflammatory cells.

In cases of peritonitis and postoperative ileus, the intestinal lumen is invaded by inflammatory cells, such as intestinal muscularis macrophages, from the outside of the intestinal wall. The intestinal muscle layer contains a dense network of ramified resident macrophages in the serosa, myenteric plexus, and interior muscle region (29, 31, 38). In tissue culture studies of the small intestinal muscle layer, which rule out the influence of infiltrating cells, LPS stimulation upregulates the expression of the cyclooxygenase (COX)-2 and iNOS genes in resident muscularis macrophages, which can impair intestinal motility by releasing NO (4, 13, 49). In the sepsis-induced ileus model, not only muscularis resident macrophages, but also infiltrating monocyte-derived macrophages and neutrophils induced motility disorder through PGs and NO (7, 37). In addition to direct exposure of endotoxin in the case of sepsis-induced ileus, these muscularis macrophages induced muscularis inflammation that resulted in motility disorder in an intestinal manipulation-mediated postoperative ileus model (20, 21, 40, 41, 50) and a chemical-induced colitis model (14, 25, 45). Therefore, muscularis macrophages are thought to play a major role in the development of intestinal motility disorders caused by various types of intestinal inflammation (1, 36).

LPS stimulation upregulates the COX-2 gene expression in macrophages (16). COX-2 is a highly inducible enzyme that catalyzes the production of PGH2 from arachidonic acid. PGH2 is converted into a series of different PGs, dependent on the profile of specific PG synthases (34, 35). PGs have short half-lives and can, therefore, affect only cells that reside locally within the vicinity of their release. Therefore, PGs produced within the intestinal muscle layer are likely to affect only cells within that layer, such as smooth muscle cells, resident macrophages, interstitial cells of Cajal, or myenteric neurons. LPS also induces iNOS gene expression via NF-κB activation through TLR-4/myeloid differentiation factor 88 signaling in macrophages (32, 54). Many reports have indicated the existence of cross talk between PG-mediated signaling and iNOS-induction signaling in different types of cells (3, 15, 48). Previously, our laboratory reported that, in rat intestinal muscularis resident macrophages, LPS-induced

Address for reprint requests and other correspondence: M. Hori, Lab. of Vet Pharmacology, The Univ. of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8602, Japan (e-mail: ahoris@aecc.u-tokyo.ac.jp).
iNOS gene expression was almost completely abolished by indomethacin; meanwhile, LPS-induced COX-2 gene expression was not abolished by Nω-nitro-L-arginine methyl ester (L-NAME), suggesting that COX-2 derived PGs are critical for LPS-induced iNOS gene expression (13). However, the details of the COX-2/PGs pathway related to iNOS induction in intestinal macrophages during inflammation remain unclear.

In the present study, we aimed to clarify the types of PGs and PG receptors that contribute to iNOS induction and the subsequent dysmotility in LPS-stimulated mouse ileum. Our findings provide new evidence to indicate that PGE2 produced by EP2 and EP4 receptors in intestinal muscularis macrophages can activate iNOS gene expression through the EP2 and EP4 receptors in intestinal muscularis macrophages.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice (8 wk old) were purchased from Charles River Japan. The op/op mice were purchased from Jackson Laboratories. EP2- or EP4-receptor knockout (KO) mice were established as previously reported (12, 18, 42). Protocols for the animal experiments and care were approved by the Institutional Review Board of the University of Tokyo (approval code: P07–084). All experiments were performed in strict compliance with the Guide for Animal Use and Care from the University of Tokyo and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Preparation of RAW264.7 cells. The RAW264.7 murine macrophage cell line (TIB-71, American Type Culture Collection, Manassas, VA) was maintained at 37°C and 5% CO2 in 10-cm dishes with DMEM (Gibco, Grand Island, NY), supplemented with antibiotics and 10% FBS. Cells in passages 5–8 were used in the experiments. Before the start of each experiment, the cells were incubated overnight in culture medium with 1% FBS.

Preparation of peritoneal macrophages. To collect peritoneal macrophages, we injected 2 ml of 10% protease peptone (BD Life Sciences, Sparks, MD) intraperitoneally into the C57BL/6J mice or the EP2 KO mice. After 48 h, the peritoneal cavity was washed with 5 ml of ice-cold phosphate-buffered saline, and the macrophages were collected. The macrophages were centrifuged, suspended in RPMI-1640 medium (Gibco), seeded onto 6-cm dishes, and allowed to adhere for 2 h. Floating cells were then washed out, and the adherent cells were used in the experiments. Before the start of each experiment, the cells were incubated overnight in culture medium with 1% FBS.

Preparation of ileal tissues. Ileal tissues were prepared as previously described (13, 14). Briefly, the ileum from each mouse 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 physiologic 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, aerated with 95% O2–5% CO2 to adjust pH to 7.3 at 37°C. LPS exposure was performed as previously described (14). Briefly, total RNA was subsequently extracted from the tissues or the cells with TRIzol (Invitrogen Japan, Tokyo, Japan), then the cells were precipitated with isopropanol, and suspended to a concentration of 1 μg/μl in RNase-free distilled water. RT-PCR was performed for evaluating the expression of DP and EP receptors. Briefly, first-strand cDNA was synthesized with random 9-mer oligonucleotide primers and avian myeloblastosis virus 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 using the “hot start” method with Taq-Gold (Perkin-Elmer, Branchburg, NJ) was conducted in the presence of the oligonucleotide primers listed in Table 1. The PCR samples were denatured initially at 95°C for 10 min, amplified at 32 cycles at 94°C for 40 s, 55°C for 1 min, and 72°C for 1 min with a thermal cycler (Takara PCR Thermal Cycler MP, Takara Biomedicals, Tokyo, Japan). The PCR products in each cycle were separated electrophoretically on a 2% agarose gel containing 0.1% ethidium bromide. To avert the risk of contaminating the DNA, we performed PCR amplification by using total RNA in the absence of the reverse transcription step as a negative control. A model FAS-III ultraviolet trans-illuminator (Toyobo, Tokyo, Japan) was used for visualizing the fluorescent bands.

Real-time qRT-PCR was performed for evaluating time-dependent changes in the expressions of iNOS and COX-2 mRNA, as previously described (46). ABI PRISM 7000 instrument (Applied Biosystems, Forester City, CA) was used for qRT-PCR amplification and detection. qRT-PCR samples were prepared in triplicate, with each sample comprising a 25-μl reaction mixture in a MicroAmp optical 96-well reaction plate sealed with an optical adhesive cover (Applied Biosystems). Each reaction well was treated with 2.5 μl of template DNA, 12.5 μl of platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Japan, Tokyo Japan), 10 pmol each of forward and reverse primers, 0.25 μl of primer mix, and 0.25 μl of platinum SYBR Green qPCR SuperMix-UDG. The PCR amplification was performed as follows: 1 cycle at 95°C for 1, 1 cycle at 95°C for 30 s, and 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR products in each cycle were separated electrophoretically on a 2% agarose gel containing 0.1% ethidium bromide. The cDNA was then purified. PCR primers were designed with PrimerBank, and the primer sequences are described in Table 1. A model FAS-III ultraviolet trans-illuminator (Toyobo, Tokyo, Japan) was used for visualizing the fluorescent bands.

Table 1. PCR primer sequences and optimal product sizes

| Gene Name | Primer Sequence | Product Size (bp) |
|-----------|-----------------|------------------|
| GAPDH     | CCCTGTTGGTTAGCGG STAT | 269 |
| iNOS      | GTTCCCTATGCACGGGTGTA | 397 |
| COX-2     | GGGCGGATCTTGTCAGCTTC | 487 |
| Mac-1     | GCCAAAGATGACGTGCTG   | 108 |
| CD11b     | CCACGCTAGGAGATGAGCT | 770 |

For real-time quantitative RT-PCR

Forward (5′ to 3′) | 3′ to 5′ | Size, bp |
|------------------|----------|----------|
| TGGTCGGACACAGCATTTGG | GGGCGGATCTTGTCAGCTTC | 487 |
| TGGTCGGAATGTTGATGG  | GGGCGGATCTTGTCAGCTTC | 487 |
| GGGCGGATCTTGTCAGCTTC | GGGCGGATCTTGTCAGCTTC | 487 |

For semiquantitative RT-PCR

Forward (5′ to 3′) | 3′ to 5′ | Size, bp |
|------------------|----------|----------|
| GCCAAAGATGACGTGCTG | GCCAAAGATGACGTGCTG | 108 |
| GCCAAAGATGACGTGCTG | GCCAAAGATGACGTGCTG | 770 |

For real-time quantitative RT-PCR

Forward (5′ to 3′) | 3′ to 5′ | Size, bp |
|------------------|----------|----------|
| GCCAAAGATGACGTGCTG | GCCAAAGATGACGTGCTG | 108 |
| GCCAAAGATGACGTGCTG | GCCAAAGATGACGTGCTG | 770 |

For semiquantitative RT-PCR

Forward (5′ to 3′) | 3′ to 5′ | Size, bp |
|------------------|----------|----------|
| GCCAAAGATGACGTGCTG | GCCAAAGATGACGTGCTG | 108 |
| GCCAAAGATGACGTGCTG | GCCAAAGATGACGTGCTG | 770 |
and 500 nM carboxy-X-rhodamine reference dye. Plasmid and genomic DNA were serially diluted 10-fold, and this step was performed in triplicate for establishing the standard calibration curves, which are constructed by plotting the threshold cycle (Ct) vs. the log concentration. For any unknown total DNA sample, the absolute quantity of both plasmid and genomic DNA was obtained by interpolating the Ct value from the sample against the standard calibration curves. A negative control was set up by substituting the template with double-distilled H2O. Repeatedly, this resulted in a high Ct value, which was taken to be the nadir, or lowest detectable range.

**Immunohistochemistry.** Mice received intraperitoneal injections of Dextran-Texas Red Beads (molecular weight 7000, 2 mg/mouse, Invitrogen, Tokyo, Japan). Twelve hours later, mice were killed, and ileum muscle strips were collected. The muscle strips were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for detecting iNOS or with Zamboni solution for detecting COX-2, and then they were processed for whole mount preparations. Samples were incubated overnight at 4°C with anti-iNOS antibody (1:500; Transduction Laboratories, Lexington, KY) or anti-COX-2 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies were detected with Alexa 568-conjugated anti-mouse IgG (1:1,000; Invitrogen, Tokyo, Japan) or FITC-conjugated anti-mouse IgG (1:200; Sigma, St. Louis, MO), respectively. Colocalization was analyzed by using a confocal laser scanning microscope (LSM510; Zeiss, Tokyo, Japan). Dextran-positive cells were considered to be intestinal resident macrophages (38).

**Measurement of muscle tension.** Longitudinal muscle tension was measured as previously described (13, 14). Briefly, muscle tension was isometrically recorded with a force displacement transducer. Muscle strips were cut into 3 × 5-mm pieces that were attached to a holder under a resting tension of 10 mN. After equilibration for 15 min in a bath, each strip was repeatedly exposed to high-K+ (72.7 mM) solution until stable responses were observed.

**NO measurement.** NO released into the culture medium was measured by monitoring the fluorescent compounds created by 3-diaminonaphthalene exposed to NO2/NO3. After FBS starvation, RAW264.7 macrophages (3 × 104 cells/well) were incubated with 1% FBS DMEM without phenol red, then the cells were stimulated with LPS (1 µg/ml) for 4–24 h. Medium from each cell-culture experiment was immediately frozen in liquid nitrogen and kept at −80°C until assayed. After the medium was thawed on ice, the amounts of NO2/NO3 were measured with the appropriate assay kits, according to the manufacturer’s instructions (Dojindo Laboratories, Kumamoto, Japan).

**Western blot analysis.** RAW264.7 cells stimulated with LPS (1 µg/ml) for 0–24 h were homogenized in homogenizing buffer to extract protein. The homogenizing buffer contained 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 5 mM sodium pyrophosphate, and 1% Triton X-100. Pefabloc protease inhibitor cocktail (Roche, Indianapolis, IL) was added to the homogenizing solution. Twenty micrograms of total protein were loaded into each lane to detect iNOS and heat shock protein (HSP)-90. Proteins were separated by electrophoresis and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked by incubation with PBS containing 5% powdered milk for 30 min at room temperature. Membranes were incubated overnight at 4°C in blocking buffer that contained anti-iNOS antibody (1:1,000, Transduction Laboratories) or anti-COX-2 antibody (1:2,000 dilutions, Cayman) or anti-HSP-90 antibody (1:1,000, Santa Cruz) as the primary antibody. Then, membranes were incubated for 1 h at room temperature with biotinylated anti-mouse IgG (for iNOS, 1:1,000, Vector Laboratories) or horseradish peroxidase anti-rabbit IgG (for COX2 and HSP-90, 1:1,000, Vector Laboratories) as the secondary antibody. Then, to detect iNOS, membranes were incubated with horseradish peroxidase-streptavidin (1:1,000, Zymed Laboratories) for 1 h at room temperature. Targeted proteins were visualized with an enhanced chemiluminescence plus Western blotting detection system (Amersham Biosciences). Bands were visualized by using an LAS-1000 mini luminescence imager (Fuji Film, Tokyo, Japan).

**Statistical analyses.** Results are expressed as means ± SE. The control and test groups were compared by using one-way ANOVA,
followed by Dunnett’s multiple-comparison test. P values < 0.05 were considered statistically significant.

RESULTS

LPS induces iNOS and COX-2 expression in intestinal resident macrophages and inhibits smooth muscle contraction. At first, we tried to identify intestinal resident macrophages that phagocytosed Texas-Red-conjugated dextran beads (molecular weight 70,000) in the ileal muscle tissue from C57BL/6J mice (wild-type) and op/op mice. We confirmed the existence of intestinal resident macrophages in the wild-type mice, but not in the op/op mice (Fig. 1A).

In the ileal muscle layer from C57BL/6J mice, LPS exposure (100 μg/ml, 4 h) upregulated iNOS and COX-2 proteins in resident macrophages in the myenteric plexus region (Fig. 1B). In wild-type mice intestine, LPS upregulated the iNOS and COX-2 mRNA expression levels (0.39 ± 0.21 to 1.13 ± 0.19, 0.93 ± 0.14 to 1.32 ± 0.2, respectively; P < 0.05, n = 4). In contrast, neither iNOS nor COX-2 mRNA expression levels were increased by LPS exposure to the ileum of op/op mice lacking intestinal resident macrophages (0.64 ± 0.24 to 0.76 ± 0.18, 0.81 ± 0.2 to 0.91 ± 0.13, respectively; n = 4) (Fig. 2, A and B, respectively). Thus LPS activates muscularis resident macrophages, resulting in the induction of COX-2 and iNOS. Figure 3, A and B, shows carbachol-induced contraction in the presence or absence of LPS treatment in the ileal tissues isolated from wild-type mice and op/op mice. LPS treatment significantly diminished the carbachol-induced contraction in wild-type mice. A NOS inhibitor, l-NAME (300 μM), recovered the muscle contractility near to the control level. In contrast, LPS did not induce dysmotility in the ileal tissues isolated from op/op mice (Fig. 3, C and D). Furthermore, pretreatments (30 min) with a selective iNOS inhibitor 1400W (1 mM) and a selective COX-2 CAY10404 attenuated LPS-induced dysmotility. In contrast, short-time treatment with CAY10404, after LPS exposure and just before the carbachol application, failed to recover the LPS-induced dysmotility (Fig. 3E), indicating that continuous PG signaling is critical for LPS-induced dysmotility.

COX-2-derived PGE2 induces iNOS expression via EP2 and EP4 receptors and attenuates ileal motility. CAY10404 (pretreatment) attenuated the LPS-induced iNOS gene expression in ileal tissue isolated from wild-type mice (48.2 ± 12.9%; n = 4) (Fig. 4A), indicating that COX-2-derived PGs participate in iNOS induction by LPS stimulus. To identify the PG species that are involved in the iNOS mRNA expression, we tested the effects of PGD2, PGE2, PGF2α, and thromboxane A2 (1 μM, 4 h) on ileal tissues. We found that PGE2 was the only PG that increased iNOS mRNA expression (0.13 ± 0.06 to 1.08 ± 0.03; n = 4) (Fig. 4A; other than PGs, data not shown). We further tested selective agonists of PGE2 receptors for their effects on iNOS induction, and we found that the EP2-selective agonist ONO-AE1–248 (1 μM) and the EP4-selective agonist ONO-AE1–329 (1 μM) induced the expression of iNOS in the ileum tissue (Fig. 4A).

We next investigated the effects of LPS on iNOS mRNA expression and intestinal dysmotility in ileal tissue from EP2 or EP4 KO mice. In intestine from wild-type mice, LPS stimulation significantly increased iNOS mRNA. In contrast, in the ileal tissue from EP2 or EP4 KO mice, the LPS-induced upregulation of iNOS gene expression was greatly reduced (Fig. 3B). In ileal tissue isolated from EP2 or EP4 KO mice, there was no effect of LPS on carbachol-induced ileal contractions (Fig. 4, C and D), which contrasted with findings in WT mice (Fig. 3C).

Estimation of mechanisms of LPS-mediated iNOS induction through PGE2/EP2 and EP4 signaling in macrophages. We next investigated possible mechanisms of LPS-mediated iNOS induction via PGE2/EP2 and EP4 signaling by using RAW264.7 macrophages, ileal resident macrophages, and peritoneal macrophages. In RAW264.7 macrophages, LPS upregulated iNOS mRNA and protein expression, which was almost completely inhibited by COX-2 inhibitor CAY10404 (Fig. 5, A and B). COX-2 and iNOS protein expressions and nitrate accumulation in the culture medium occurred in a time-dependent manner (Fig. 5, B and C).

To strengthen our hypothesis that LPS induces NO in ileum macrophages via EP2/EP4 receptors, ileal resident macrophages were isolated by FACS analysis against CD11b, a major cell-surface marker of macrophages (Fig. 6A). In isolated ileal resident macrophages, LPS also upregulated iNOS mRNA expression, which was almost completely inhibited by COX-2 inhibitor CAY10404 (Fig. 6B). PGE2 significantly upregulated iNOS mRNA expression. EP2-selective agonist (ONO-AE1–259, 1 μM) and EP4-selective agonist (ONO-AE1–329, 1 μM), but not EP1- and EP3-selective agonists (ONO-DI-004 and ONO-AE248, 1 μM) enhanced iNOS mRNA expression (Fig. 6B). Conversely, in RAW264.7 cells, BW245C [DP (DP1) selective agonist, 1 μM], DK-PGD2 [CRTH2 (DP2) selective agonist, 1 μM], and PGF2α (EP selective agonist, 1 μM) did not increase iNOS gene expression.
Peritoneal macrophages purified from EP2 KO mice only weakly upregulated iNOS gene expression upon stimulation with LPS compared with wild-type peritoneal macrophages (Fig. 6C). These results indicate that LPS induces iNOS in ileal resident macrophages produced via PGE2/EP2 signaling. As EP2 and EP4 receptors have been reported to couple with Gs signaling, we next investigated the effects of modulators of cAMP/PKA signaling on iNOS gene expression using RAW264.7 macrophages. A membrane-permeable cAMP derivative, dibutyryl-cAMP, and an adenylate cyclase activator, forskolin, dose-dependently upregulated iNOS gene expression (Fig. 7A). In addition, a selective PKA inhibitor, KT5720, inhibited iNOS gene expression in the LPS-stimulated macrophages (Fig. 7B).

Finally, we examined the effects of KT5720, ERK inhibitor (PD-98059), and phosphatidylinositol 3-kinase (PI3K) inhibitor (wortmannin) on EP2 and EP4 agonist-induced iNOS gene expression (Fig. 7C). We found that KT5720 and PD-98059, but not wortmannin, significantly inhibited iNOS gene expression stimulated by EP2 and EP4 agonists.
DISCUSSION

Intestinal inflammation is associated with dysfunction from the mucosal barrier, which can occur in intestinal bowel diseases, allergic diarrhea, and infectious enteritis, or conditions that affect muscular or outer layers of the bowel wall, such as peritonitis and postoperative ileus. In the latter case, intestinal muscularis macrophages play an important role in inducing intestinal dysmotility during inflammation by producing PGs and NO (7, 20, 40, 41, 50). However, the roles of PGs in iNOS induction and the subsequent intestinal dysmotility are not well understood. In the present study, we demonstrate that PGE\(_2\) produced due to LPS stimulation activates the muscularis resident macrophages via EP\(_2\) and EP\(_4\) receptors to express the iNOS gene, which, in turn, induces intestinal dysmotility via the production of NO. PGE\(_2\) induces iNOS gene expression through the cAMP/PKA/MAPK signal transduction pathways in the intestinal muscularis macrophages.

Intestinal muscularis macrophages play an important role in inducing intestinal dysmotility during inflammation by producing PGs and NO (7, 20, 41, 50). Indeed, LPS upregulates the gene expression of COX-2 and iNOS in the intestinal muscle layer. Our laboratory’s previous work also showed that LPS stimulation upregulated COX-2 and iNOS protein in ED\(_2\)-positive resident macrophages in rat (8, 13). In the present study, immunohistochemical analysis revealed that the COX-2 and iNOS genes were expressed in intestinal resident macrophages that are ramified shape, able to phagocytose dextran. In ileal tissue isolated from macrophage colony-stimulating factor-deficient mice (op/op mice), LPS could not induce the expression of the COX-2 and iNOS genes. As muscularis resident macrophages do not develop in the intestinal muscle layer of op/op mice (30), we concluded that LPS can directly activate muscularis resident macrophages to induce COX-2 and iNOS gene expression in mouse small intestine, which is consistent with a previous report (51). A limitation of the present study is that the anti-F4/80 antibody was not available for isolation of ileal muscularis resident macrophages, because the antibody was not efficient without paraformaldehyde fixation. At present, cellular biological characters of ileal muscularis macrophages are still unclear, whereas CD11b (47), CD11c (9), and CD163 (52) are also well known as a marker of resident macrophages. Our results indicate that LPS stimula-
tion enhanced iNOS mRNA expression in a COX-2/PGE2-dependent manner in the isolated CD11bHigh cells. These evidences lead us to conclude that isolated CD11bHigh cells are ileal muscular resident macrophages.

Although the activation of TLR-4 by LPS can directly activate NF-κB via myeloid differentiation factor 88/IL-1 receptor-associated kinase-1/tumor necrosis factor receptor-associated factor signaling (19, 28) that induces iNOS gene expressions in various immune cells, iNOS induction by LPS in intestinal muscularis macrophages may be mediated indirectly through the production of PGs, because the LPS-induced iNOS gene expression was almost completely inhibited by a COX-2 selective inhibitor, CAY10404. It has been reported that a COX-2 inhibitor ameliorated intestinal inflammation and associated intestinal dysmotility, resulting in iNOS suppression in an LPS-induced sepsis model (1, 26, 40). However, the type of PG and the receptor that contribute to iNOS gene expression in the muscularis macrophages during LPS stimulus was not previously determined. Our pharmacological analysis indicated that only PGE2 can activate the macrophages via EP2 and EP4 receptors to upregulate the expression of the iNOS mRNA (Fig. 4, A and B). Our laboratory’s previous study provided evidence that LPS induces PGE2 in rat ileal muscle tissue (13). Surgical manipulation also markedly increased the PGE2 level in the peritoneal cavity (26). This study examined the effects of LPS on iNOS gene expression in intestines of EP2 or EP4 KO mice, and we found that LPS-activated iNOS gene induction was less than that of wild-type mice. Although we could not study EP2 and EP4 double-KO mice, it is highly possible that LPS indirectly induces iNOS gene expression both via PGE2/EP2 and PGE2/EP4 pathways in the intestinal macrophages.
Fig. 6. PG signaling for iNOS mRNA induction in ileal muscularis resident macrophages. 

**A**: fluorescence-activated cell sorting (FACS) detection of CD11b-positive resident macrophages in an enzymatically digested ileum cell suspension from C57BL/6J. CD11b<sup>High</sup> population (in the circle) was isolated. SSC, side scatter; PE, phycoerythrin.

**B**: effect of LPS and EP agonists on iNOS mRNA expression in isolated resident macrophages. LPS (1 μg/ml) stimulation for 4 h was used as a positive control. *P < 0.05 compared with resting macrophages. We used the concentration of each EP agonist (1 μM) that induces submaximum responses in biological reactions, as determined from published references. EP<sub>1</sub>, ONO-DI-004; EP<sub>2</sub>, ONO-AE1–259; EP<sub>3</sub>, ONO-AE-248; and EP<sub>4</sub>, ONO-AE1–329.

**C**: effect of LPS stimulation (1 μg/ml, 4 h) in the peritoneal macrophages isolated from EP<sub>2</sub> KO mice. Each bar shows mean ± SE of 4 real-time RT-PCR analyses.

---

Fig. 7. PKA signaling for iNOS mRNA induction in LPS-stimulated macrophages. All experiments were analyzed from semi-quantitative RT-PCR. 

**A**: effect of dibutyryl (db)-cAMP (1 and 10 μM) and forskolin (1 and 10 μM) on iNOS mRNA expression in RAW264.7 macrophages at 4 h after the stimulation. Macrophages stimulated with LPS (1 μg/ml, 4 h) were used as a positive control.

**B**: effect of protein kinase A inhibitor, KT5720 (0.01–10 μM), on the 1 μg/ml LPS-induced iNOS mRNA expression in RAW264.7 macrophages. KT5720 was applied 30 min before the LPS stimulation.

**C**: effects of various protein kinase inhibitors on EP<sub>2</sub> and EP<sub>4</sub> selective agonist (1 μM)-induced iNOS mRNA expressions. An ERK inhibitor, PD-98059 (1 μM), and a phosphatidylinositol 3-kinase inhibitor, wortmannin (WM; 100 nM), were added 30 min before the EP<sub>2</sub> and EP<sub>4</sub> agonist, ONO-AE1–259 and ONO-AE1–329, respectively. Each bar shows the mean ± SE of 4 experiments. *P < 0.05 compared with resting macrophages. #P < 0.05 compared with each agonist stimulation (1 mg/ml, 4 h).
It has been reported that NO itself is able to enhance PG production via COX nitrosylation and/or COX upregulation (33). However, in our laboratory’s previous report (13), Nω-monomethyl-L-arginine did not interfere with LPS-induced COX-2 expression or iNOS expression in rat ileal tissue. Taken together, in ileal smooth muscle tissue, these findings suggest NO may not affect PGs production, and that other components of LPS-induced intestinal inflammatory cascade induce PGs.

To clarify the intracellular signaling underlying iNOS induction via PGE2, we further determined the effect of LPS on iNOS induction using RAW264.7 macrophages and purified peritoneal macrophages. In RAW264.7 macrophages, we confirmed that LPS induced iNOS mRNA and protein expression in a time-dependent manner, and that this induction of iNOS was abolished by COX-2 inhibitor. We also measured the levels of nitrites in the culture medium of macrophages stimulated with LPS and found that nitrites accumulated in the medium. In peritoneal macrophages isolated from EP2 KO mice, LPS-induced iNOS gene expression was significantly inhibited in a manner similar to the inhibition in mouse intestinal tissue. These results indicate that RAW264.7 macrophages and intestinal muscularis macrophages use the same EP receptor signaling pathway to induce iNOS mRNA in response to LPS.

The iNOS upregulation due to LPS, EP2 agonist, and EP4 agonist were all attenuated by PKA inhibitor KT-5720. In addition, a membrane-permeable cAMP analog, dibutylryl-cAMP, and an adenylyl cyclase activator, forskolin, increased iNOS gene expression in a concentration-dependent manner. These results suggest that cAMP/PKA signaling predominantly contributes to the LPS-induced iNOS expression through the EP2 and EP4 receptors. In fact, EP2 and EP4 receptors bind to Gs protein in a human embryonic kidney cell expression system (35, 39, 44). Some studies have examined the downstream signaling cascades from PKA activation to iNOS expression. In human oral squamous cell carcinoma SCC-9 cells, PGE2/EP2 signaling enhanced iNOS expression via transactivation with the EGF receptor through Src activation (6). In RAW264.7 macrophages, lipoteichoic acid also upregulated iNOS through PGE2/EP2 signaling following p38 MAPK and ERK activation, but not JNK activation (3, 17). On the other hand, the downstream signaling of EP2 is mediated mostly by PKA, while the downstream signaling of EP4 is mediated equally by PKA and PI3K (10, 11, 39). Thus iNOS expression can be induced via PKA and PI3K. LY-294002, a PI3K inhibitor, has been shown to inhibit LPS-induced NO production in RAW264.7 cells (23, 24). In the present study, we found that ERK inhibitor, but not PI3K inhibitor, partially inhibited EP2- and EP4-induced iNOS gene expression, suggesting that at least the ERK pathway may contribute to the PGE2/EP2 or PGE2/cAMP/PIKA signaling cascade. Further studies are required to establish which cascade is most effective for PGE2-mediated iNOS induction in the intestine.

The present study clearly indicates that LPS exposure activates intestinal muscularis resident macrophages to produce PGE2, which, in turn, upregulates iNOS expression in autocrine and paracrine manners via EP2/EP4 receptors to induce intestinal dysmotility. In the clinical condition, COX-2-mediated PGE2 and NO produced by iNOS from infiltrated monocyte-derived macrophages and leukocytes may also be important factors in the induction of intestinal dysmotility. In fact, intestinal manipulation-mediated muscularis inflammation is reduced in COX-2-deficient mice compared with wild-type mice, and intestinal dysmotility is ameliorated by COX-2 inhibitor (1, 26). These results indicate that nonsteroidal anti-inflammatory drugs (NSAIDs) could ameliorate intestinal dysfunction. However, NSAIDs cause intestinal damage, such as hemorrhagic lesions, because of the depletion of endogenous physiologically functional PGE2 (2, 53). Therefore, selective COX-2 inhibitors or selective PGE2 receptor antagonists may be effective treatments for intestinal inflammation with motility dysfunction. Kunikata et al. (27) studied the effects of indomethacin in a small intestinal ulceration animal model and found that intestinal hemorrhagic lesions induced by NSAIDs are caused by the inhibition of EP3/EP4 receptors. On the other hand, EP1 receptor appears to play an important role in the growth suppression of colon carcinogenesis (43). These results indicate that EP2 receptor blockade may be a useful therapeutic strategy for the treatment of intestinal dysmotility due to muscularis inflammation.

In summary, we clarified that PGE2 produced by LPS stimulus activates the muscularis resident macrophages via EP2 and EP4 receptors to express the iNOS gene, which, in turn, induces intestinal dysmotility via the production of NO. PGE2 induces iNOS gene expression through the cAMP/PKA/MAPK signal transduction pathways in the intestinal muscularis macrophages.

ACKNOWLEDGMENTS

We thank Ono Pharmaceutical Co. Ltd. for supplying ONO DI-004, ONO-AE1-259, ONO-AE-248, and ONO-AE1-329.

GRANTS

This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education (to M. Hori, nos. 18380173 and 21380178; to H. Ozaki, no. 20228005; and to T. Murata, no. 19688014), the Yakult Bioscience Foundation (H. Ozaki), and the 2008 Strategic Research Base Development Program for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan (T. Tajima).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.T., T. Murata, K.A., M.M., and T. Murata performed experiments; T.T., T. Murata, K.A., Y.U., M.M., T. Matsuoka, and S.N. analyzed data; T.T., T. Murata, K.A., Y.U., M.M., T. Matsuoka, S.N., H.O., and M.H. interpreted results of experiments; T.T. and M.M. prepared figures; T.T. drafted manuscript; T.T. and M.H. edited and revised manuscript; T.T. and M.H. approved final version of manuscript; T. Murata, K.A., Y.U., T. Matsuoka, S.N., H.O., and M.H. conception and design of research.

REFERENCES

1. Bauer AJ, Boeckxstaens GE. Mechanisms of postoperative ileus. Neurogastroenterol Motil 16, Suppl 2: 54–60, 2004.
2. Bjarnason I, Zanelli G, Smith T, Prouse P, Williams P, Smethurst P, Delacey G, Gumpel MJ, Levi AJ. Nonsteroidal antiinflammatory drug-induced intestinal inflammation in humans. Gastroenterology 93: 480–489, 1987.
3. Chang YC, Li PC, Chen BC, Chang MS, Wang JL, Chiu WT, Lin CH. Lipoteichoic acid-induced nitric oxide synthase expression in RAW264.7 macrophages is mediated by cyclooxygenase-2, prostaglandin E2, protein kinase A, p38 MAPK, and nuclear factor-kappaB pathways. Cell Signal 18: 1235–1243, 2006.
4. De Winter BY, Breedenoord AJ, De Man JG, Moreels TG, Herman AG, Pelckmans PA. Effect of inhibition of inducible nitric oxide synthase
and guanylyl cyclase on endothrin-induced delay in gastric emptying and intestinal transit in mice. Shock 18: 125–131, 2002.
5. Deitch EA, Xu D, Franko L, Ayala A, Chaudry IH. Evidence favoring the role of the gut as a cytokine-generating organ in rats subjected to hemorrhagic shock. Shock 1: 141–145, 1994.
6. Donnini S, Finetti F, Solito R, Terzulli E, Sacchetti A, Morbidelli L, Patrignani P, Ziche M. EP3: prostaglandin receptor promotes squamous cell carcinoma growth through epidermal growth factor receptor transactivation and iNOS and ERK1/2 pathways. FASEB J 21: 2418–2430, 2007.
7. Eskandari MK, Kalf JC, Billiar TR, Lee KK, Bauer AJ. Lipopolysaccharide activates the muscularis macrophage network and suppresses circular smooth muscle activity. Am J Physiol Gastrointest Liver Physiol 273: G727–G734, 1997.
8. Eskandari MK, Kalf JC, Billiar TR, Lee KK, Bauer AJ. LPS-induced muscularis macrophage nitric oxide suppresses rat jejunal circular muscle activity. Am J Physiol Gastrointest Liver Physiol 277: G478–G486, 1999.
9. Flores-Langarica A, Meza-Perez S, Calderon-Amador J, Estrada-Garcia T, Macpherson G, Lebecke S, Saeland S, Steinman RM, Flores-Romo LF. Network of dendritic cells within the muscular layer of the mouse intestine. Proc Natl Acad Sci USA 102: 19039–19044, 2005.
10. Fujino H, West KA, Regan JW. Phosphorylation of glycosyn synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP2 and EP4 prostaglandin receptors by prostaglandin E2. J Biol Chem 277: 2614–2619, 2002.
11. Fujino H, Xu W, Regan JW. Prostaglandin E2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostaglandin receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. J Biol Chem 278: 12151–12156, 2003.
12. Hizaki H, Sugi T, Sugiymoto Y, Hirose M, Saji T, Ushikubi F, Matsutoka T, Noda Y, Tanaka T, Yoshida N, Narumiya S, Ichikawa A. Abortion of expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). Proc Natl Acad Sci USA 96: 10501–10506, 1999.
13. Hori M, Kita M, Torihashi S, Miyamoto S, Won KJ, Sato K, Ozaki H, Karaki H. Upregulation of iNOS by COX-2 in muscularis resident macrophage of rat intestine stimulated with LPS. Am J Physiol Gastrointest Liver Physiol 280: G930–G938, 2001.
14. Hori M, Nobe H, Horiguchi K, Ozaki H. MCP-1 targeting inhibits muscularis macrophage recruitment and intestinal smooth muscle dysfunction in colonic inflammation. Am J Physiol Cell Physiol 294: C391–C401, 2008.
15. Hsiao HY, Mak OT, Yang CS, Liu YP, Fang KM, Tzeng SF. TNF-alpha/INF-gamma-induced iNOS expression increased by prostaglandin E2 in rat primary astrocytes via EP2-evoked cAMP/PKA and intracellular calcium signaling. Glia 55: 214–223, 2007.
16. Hwang D. Modulation of the expression of cyclooxygenase-2 by fatty acids mediated through toll-like receptor 4-derived signaling pathways. FASEB J 15: 2556–2564, 2001.
17. Jung WK, Choi I, Lee DY, Yea SS, Choi YH, Kim MM, Park SG, Seo SK, Lee SW, Lee CM, Park YM, Choi IW. Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-kappaB pathways. Int J Biochem Cell Biol 40: 2572–2582, 2008.
18. Kabashima K, Taji S, Murata T, Nagamaichi M, Matsutoka T, Sugi T, Tsuboi K, Sugimoto Y, Kobayashi T, Miyachi Y, Ichikawa A, Narumiya S. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. J Clin Invest 109: 883–893, 2002.
19. Kaisha T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol 117: 979–987; quiz 988, 2006.
20. Kalf JC, Carlos TM, Schraut WH, Billiar TR, Simmons RL, Bauer AJ. Surgically induced leukocyte infiltrates within the rat intestinal muscularis mediate postoperative ileus. Gastroenterology 117: 378–387, 1999.
21. Kalf JC, Schraut WH, Billiar TR, Simmons RL, Bauer AJ. Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. Gastroenterology 118: 316–327, 2000.
22. Kawai T, Akira S. Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med 13: 460–469, 2007.
23. Kim Y, Moon JS, Lee KS, Park SY, Cheong J, Kang HS, Lee HY, Kim HD. Ca2+/calmodulin-dependent protein phosphatase calcineurin mediates the expression of iNOS through IKK and NF-kappaB activity in
46. Tajima T, Murata T, Aritake K, Uraide Y, Hirai H, Nakamura M, Ozaki H, Hori M. Lipopolysaccharide induces macrophage migration via prostaglandin D(2) and prostaglandin E(2). J Pharmacol Exp Ther 326: 493–501, 2008.

47. Takada Y, Hisamatsu T, Kamada N, Kitazume MT, Honda H, Oshima Y, Saito R, Takayama T, Kobayashi T, Chinen H, Mikami Y, Kanai T, Okamoto S, Hibi T. Monocyte chemoattractant protein-1 contributes to gut homeostasis and intestinal inflammation by composition of IL-10-producing regulatory macrophage subset. J Immunol 184: 2671–2676, 2010.

48. Timoshenko AV, Lala PK, Chakraborty C. PGE2-mediated upregulation of iNOS in murine breast cancer cells through the activation of EP4 receptors. Int J Cancer 108: 384–389, 2004.

49. Torihashi S, Ozaki H, Hori M, Kita M, Ohota S, Karaki H. Resident macrophages activated by lipopolysaccharide suppress muscle tension and initiate inflammatory response in the gastrointestinal muscle layer. Histochem Cell Biol 113: 73–80, 2000.

50. Turler A, Kalff JC, Moore BA, Hoffman RA, Billiar TR, Simmons RL, Bauer AJ. Leukocyte-derived inducible nitric oxide synthase mediates murine postoperative ileus. Ann Surg 244: 220–229, 2006.

51. Wehner S, Behrendt FF, Lytenski BN, Lysson M, Bauer AJ, Hirner A, Kalff JC. Inhibition of macrophage function prevents intestinal inflammation and postoperative ileus in rodents. Gut 56: 176–185, 2007.

52. Wehner S, Buchholz BM, Schuchtrup S, Rocke A, Schaefer N, Lysson M, Hirner A, Kalff JC. Mechanical strain and TLR4 synergistically induce cell-specific inflammatory gene expression in intestinal smooth muscle cells and peritoneal macrophages. Am J Physiol Gastrointest Liver Physiol 299: G1187–G1197, 2010.

53. Whittle BJ. Temporal relationship between cyclooxygenase inhibition, as measured by prostacyclin biosynthesis, and the gastrointestinal damage induced by indomethacin in the rat. Gastroenterology 80: 94–98, 1981.

54. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 269: 4705–4708, 1994.