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FULL PAPER *Pharmacology*

Phagocytotic Activation of Muscularis Resident Macrophages Inhibits Smooth Muscle Contraction in Rat Ileum

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ABSTRACT. Intestinal muscularis resident macrophages distributed in myenteric region may play an important role in the immunological host defense against infection. In this study, we investigated the phagocytic stimulation of resident macrophages on cyclooxygenase-2 (COX-2) expression and smooth muscle contraction in the small intestine of rat. After the injection of FITC-dextran to rat, phagocytosed macrophages could be detected in the myenteric plexus. FITC-positive macrophages were also immunostained with COX-2 antibody. The number of COX-2 immunopositive cells increased in a time-dependent manner reaching its maximum at 4 hr after the injection, which then decreased gradually but considerable number of cells were still remained on 7 days. The injection of FITC-dextran, however, did not change the population of ED2-positive resident macrophages even on 7 days. Production of PGE_2 was significantly higher in the dextran treated tissue as compared to control tissue. In the smooth muscle tissue phagocytosed dextran, carbachol-induced contraction was significantly decreased. The suppression of the carbachol-induced contraction was completely restored by COX inhibitor, indomethacin. Finally we demonstrated that, in freshly isolated macrophage cells, addition of dextran induced a slow and sustained increase in intracellular Ca^{2+} concentration. These results indicate that phagocytotic activation of muscularis resident macrophages induces COX-2 gene expression and then results in production of PGE_2 to suppress the smooth muscle contractile activity.
KEY WORDS: intestinal smooth muscle, phagocytosis, prostaglandin, resident macrophage.

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In a host defense system, macrophages play an important role as a first line of defense against microbial infections [27]. In the digestive system, the gut mucosa contains a large population of macrophages that provide a specific immune response against the lumenal milieu. Macrophages occurring in specific sites in normal noninflamed tissues are termed resident macrophages. They are distributed through most tissues of the body and are one of the first cell types to sense microbial invaders. Recently, the resident macrophages have also been identified in the muscularis externa of the gastro-intestinal (GI) tract. They are regularly distributed in the subserosa and at the level of the myenteric plexus [14]. Resident macrophages at the level of the myenteric plexus are closely come into view not only to myenteric neurons but also proximity to the interstitial cells of Cajal (ICCs), which are considered to be the pacemaker cells in GI tract [15, 22, 23]. The close proximity of macrophages to ICCs may lead to effects on the motility of GI tissues. Very recently it is suggested that the activation of resident macrophages is related to the alterations of the ICCs and myenteric nerves of rat intestine with colitis [10].

It has been reported that intraperitoneal injection of lipopolysaccharide (LPS) led to an inhibition of contractility of intestinal smooth muscle by the activation of macrophages [3]. And incubation of isolated intestinal tissues with LPS decreased smooth muscle contractions and enhanced the expression of inducible nitric oxide synthase (iNOS) and cyclooxigenase-2 (COX-2) [6, 28]. These findings suggest that the intestinal resident macrophages can control smooth muscle contraction without recruitment of extrinsic immune cells. On the other hand, macrophages possess a number of phagocytic receptors that mediate opsonic and non-opsonic uptake by binding to specific molecular components of microbial surfaces [5, 30]. Phagocytosis of microorganisms is accompanied by the activation of Toll-like receptors, which participate in promoting inflammatory responses [16, 29].

Several signaling pathways related to prostaglandin synthesis have been suggested in the macrophages during the phagocytotic stimulations. The production of prostaglandin E_2 (PGE_2) by mouse peritoneal macrophages occurs within minutes of intraperitoneal zymosan administration and plays a role in mediating early phases of the immune responses [11, 13]. It has also been reported that the non-receptor mediated phagocytosis of particles such as latex beads leads to the prompt induction of PGE_2 in bone-marrow derived macrophage [24]. On the other hand, PGE_2 which is released from peritoneal macrophages has been demonstrated to produce hyperalgesia [4] and increased vascular permeability [11]. We have previously reported that the injected dextrans, phagocytosed by resident macrophages reside in the intestinal muscle layer [20, 26, 28]. However, the possible changes in the macrophages and smooth muscle functions after the phagocytotic stimulation have not been elucidated. In the present study, the experiments are performed to assess whether the phagocytosis of exogenous

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particles produces COX-2 expression and secondary changes to smooth muscle contractile function.

MATERIALS AND METHODS

Animals and tissue preparations: Wistar rats either sex (150–250 g) were anesthetized and given injections of dextran (MW 70,000) labeled with or without FITC (0.25 mg/g body weight) intravenously or intraperitoneally (dextran-injected rat). They were sacrificed at 0 hr, 4 hr, 12 hr, or 7 days after injections. As controls, same injections were carried out using same amount of physiological saline (control rat). In some experiments, rats were used as control without injection of the physiological saline. The rats were sacrificed and the excised terminal ileum was placed in physiological salt solution (PSS) (in mM): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.5, $NaHCO_3$ 23.8, glucose 5.5 (pH 7.4 at 37°C) aired with 95% O_2 and 5% CO_2. Animal care and treatment were conducted in conformity with The Institutional-Guidelines of Yamaguchi University.

Measurement of muscle tension: Muscle strips of terminal ileum from the rat injected intravenously with non-labeled dextran were suspended along their circular axis in a tissue bath filled with PSS at 37°C in an atmosphere of 95% O_2 and 5% CO_2. The response of the strips was measured isometrically under the resting tension of 10 mN and recorded. Each strip was repeatedly exposed to 65.4 mM KCl until responses became stable. Concentration-response curves were obtained by the cumulative application of agonist. On the other hand, single dose application was adapted for the measurement of absolute force. At the end of tension measurements, the wet weight of each muscle strip was measured. In some experiments, the contraction was normalized by the milligram of wet weight tissue (mN/mg wet wt).

Immunohistological examination: Rats were injected intravenously with FITC-dextran and sacrificed. For immunohistochemistry, tissues were fixed with Zamboni's solution for 4 hr for anti-macrophage antibody (ED2; Serotec, Oxford, England) or anti-COX-2 antibody (Santa Cruz Biotech, CA, U.S.A.). Muscle coats were separated, cut into small pieces and incubated overnight at 4°C with first antibodies (ED2, 1:500 and COX-2, 1:200). Samples were treated with biotinylated secondly antibodies and detected with Texas red-conjugated streptavidin (1:100, Vector). Samples were examined with a confocal laser scanning microscope (MRC-1024; BioRad, CA U.S.A.). Some were treated with Vector Stain ABC kit (Vector, CA U.S.A.) and detected with enzyme-immunohistochemistry using 0.1% diaminobenzidine tetrahydrochloride containing 0.02% H_2O_2. Immunopositive cells were counted and tabulated (per mm^2) in each sample (n=3).

Cell preparation and measurement of intracellular Ca^{2+} concentration: Resident macrophages were isolated from the intestinal muscularis by standard collagenase treatment method as previously described [20]. Freshly isolated macrophages on glass cover slips were loaded with fura-PE3 by exposure to a HEPES buffer solution (in mM: NaCl 125.4, glucose 11.5, KCl 5.9, $MgCl_2$ 1.2, HEPES 10, pH 7.4, either $CaCl_2$ 1.5 or EGTA 0.5) containing 5 μM fura-PE3 acetoxymethyl ester with 0.01% cremophor EL for 30–40 min in a dark room. For fluorescence measurements, cells on glass cover slips were placed in a bath on the stage of an inverted microscope (Nikon, Tokyo, Japan) equipped with a 40-fold objective lens. The acquisition and analysis were done with a Ca^{2+}-imaging system (PTI-4700, Photon Technology International, NJ, U.S.A.). Images of 510 nm fluorescence were captured every 3 sec using 340 and 380 nm wavelength light, and the images at 340 nm were divided by the images at 380 nm to provide resultant ratio images (R340/380) that are indicators of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). All experiments were performed at 37°C [18].

Measurement of released prostaglandin E_2: Each muscle strip of terminal ileum taken from the rat injected with or without FITC-dextran was attached to a holder in an organ bath (2 m*l*) containing PSS and equilibrated for 30 min at 37°C. After incubation with PSS, 50 μl of PSS solution were removed, and the released PGE_2 were measured using an enzyme immunoassay system (Amersham Pharmacia Biotech, Tokyo, Japan). Released PGE_2 were calculated using the standard assay in the kit and expressed as picograms per milligram of wet weight tissue (*p*g/mg wet wt).

Chemicals: Other chemicals used were carbachol and indomethacin (Sigma, Tokyo, Japan), FITC-dextran, Texas-Red-dextran and dextran amino (70,000 M.W.) (Molecular Probes, OR, U.S.A.), fura-PE3 acetoxymethyl ester (Teflab, TX, U.S.A.), cremophor EL (Nacalai Tesque, Tokyo, Japan), PGE_2 (WAKO Pure Chemical, Osaka, Japan).

Statistical analyses: The data were expressed as means ± SE mean. They were analyzed using the unpaired Student's *t*-test for comparisons between pairs of groups and by one-way analysis of variance (ANOVA) followed by either Dunnett's test or the Tukey test for comparisons among more than two groups. A value of $p<0.05$ was taken as significant.

RESULTS

Induction of COX-2 in the FITC-phagocyted cells: In the present experiments, rats were injected with FITC-dextran intraperitoneally 4 hr before sacrifice. The fluorescent image of FITC-dextran was detected in the resident macrophages at the level of myenteric plexus in rat small intestine (Fig. 1A). Expanded Fig. 1A shows that FITC-dextrans phagocytosed with macrophages were localized in their phagosomes (Fig. 1B). Macrophages also expressed COX-2 proteins which were expressed evenly in the cytoplasm (Fig. 1C). Figure 1D shows colocalization of FITC-dextran and COX-2 in the same cells. Although significant immunoreactivity for COX-2 was demonstrated in the resident macrophages, neither anti-iNOS antibody nor anti-MHC class II antibody (OX6) stained these macrophages (data not shown).

Immunohistochemistry of COX-2: Muscularis macroph-

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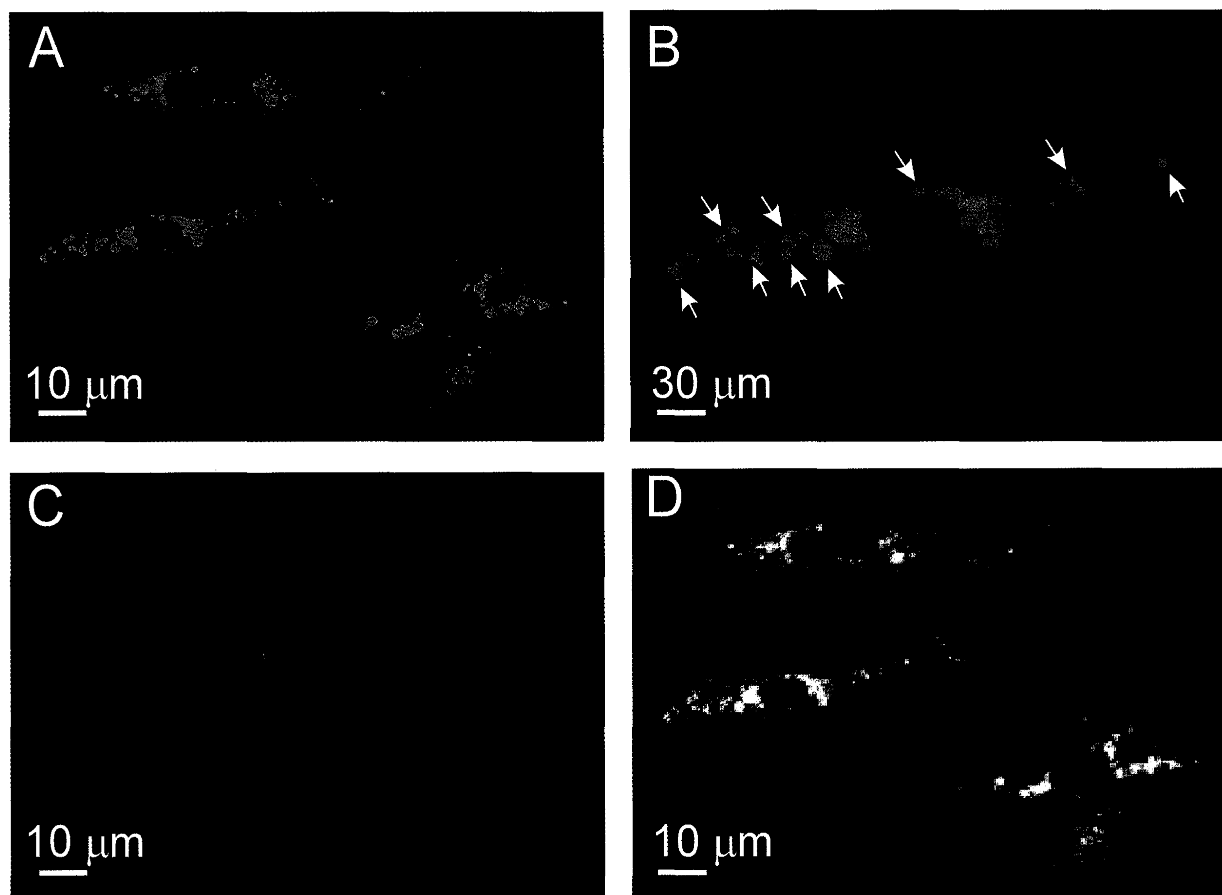

Fig. 1. Confocal micrographs of dextran-phagocytosed (A, B) and COX-2-immunopositive cells (C) at the level of the myenteric plexus in the rat ileum after 4 hr injection of FITC-dextran. A: Cells absorbed the FITC-dextran. B: Expanded Fig. 1A. C: COX-2-immunopositive cells indicated by TexasRed-fluorescence. D: Merged image of A and C. Typical results out of 3 separate experiments are shown. Scale bars, 10 μm (A, C, D), 30 μm (B). Arrows indicate the dextran particles in their phagosomes (B).

ages were labeled with anti-resident macrophage antibody (ED2). As shown in Fig. 2A, a great number of ED2 positive macrophages were distributed almost evenly in the muscle layer at myenteric plexus in the control rat. Macrophages showed multipolar and unique appearance. They protruded several processes with branches in every direction and had narrow perinuclear cytoplasm.

In control rat ileum, whole mount preparation of muscle layer stained with anti-COX-2 antibody did not indicate any immunopositive cells. The injection of FITC-dextran induced the expression of COX-2 protein and the number of COX-2 positive cells increased time dependently (Fig. 2B, C and D). COX-2 immunoreactivity was restricted in a part of the cytoplasm and did not stain whole cytoplasm. Whole mount preparation of immunostained muscle layer allowed us to count the population of immunopositive cell. Analysis of the data indicated that immunoreactivity of COX-2 positive cells were almost absent in the control rat ileum; however, the number of COX-2 immunopositive cells increased and reached maximum at 4 hr after injection of FITC-dextran, which decreased gradually but still remained high after 7 days (Fig. 2E). On the other hand, the population of entire resident macrophages in control rat ileum indicated by ED2 was 224 ± 12 cells/mm^2. The injection of FITC-dextran did not change the population of these cells even after 7 days.

Effects of the injection of FITC-dextran on PGE_2 release: Because the injection of dextran induces the expression of COX-2 protein in myenteric resident macrophages, we next examined the amount of prostaglandin E_2 (PGE_2) released from the ileal smooth muscle tissue using an enzyme immunoassay method (Fig. 3). In the control rat ileum, a certain level of PGE_2 was released spontaneously. Four hours after the injection of FITC-dextran, released PGE_2 was increased significantly. The amount of PGE_2 was returned to control level 7 days after the injection.

Effects of the injection of dextran on muscle contractions: We examined the effects of injection of dextran on smooth muscle contractility. In control rat ileum, cumulative application of carbachol (1 nM - 10 μM) induced concentration-dependent contractions. After the addition of indomethacin (10 μM) for 10 min, same concentrations of carbachol were applied again; however, the concentration-response relationship was not changed (Fig. 4A). In dextran-injected rat ileum, on the other hand, carbachol-induced contractions

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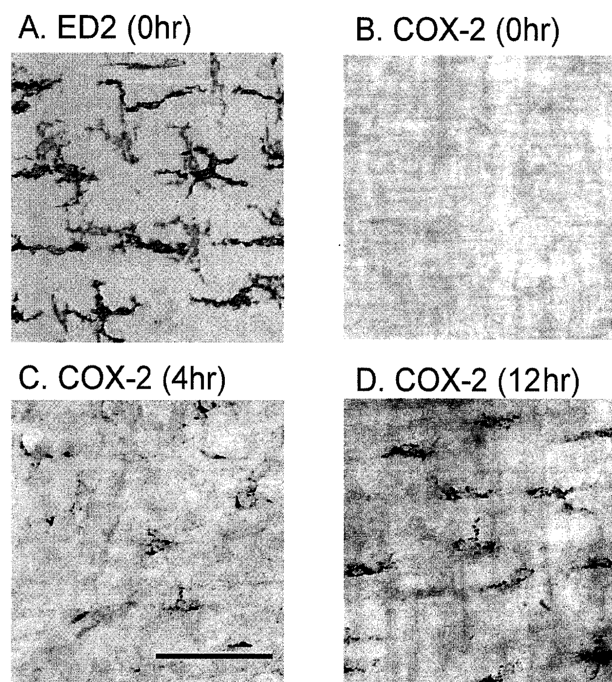

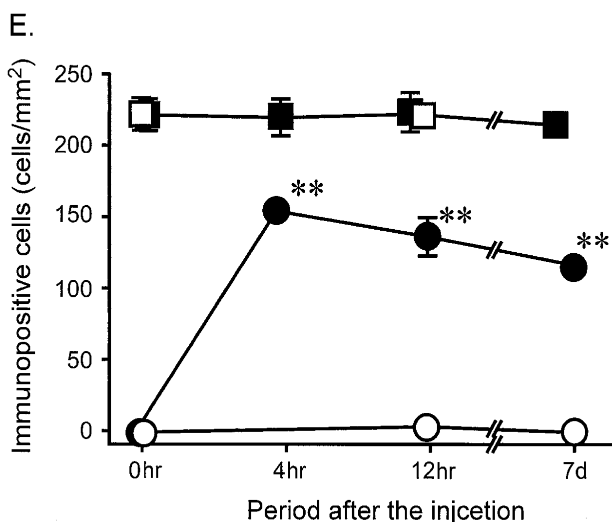

Fig. 2. Immunohistochemistry of anti-ED2 antibody (A) and anit-COX-2-antibody (B-D) in rat ileal smooth muscle layer. ED2 reactive cells are uniformly distributed in the muscle layer in the rat before injection of the dextran as shown by panel A. Before injection of the FITC-dextran, there was no expression of COX-2 reactivity in the whole mount preparations as shown by panel B. The panel C and D show cells positive for COX-2 antibody at 4 and 12 hr respectively after injection of FITC-dextran. Typical results are shown from 3 experiments. Scale bar, 100 μm (C). The panel E shows the kinetics of COX-2 and ED2-immunopositive macrophages after the injection of FITC-dextran. The populations of ED2-immunopositive macrophages are constant and there are no differences between dextran-injected (closed square) and control rat (open square). The densities of COX-2-positive cells do not change in control rat (open circle). The population increases in a time-dependent manner in dextran-injected rat (closed circle). Results are means ± SE of mean of 3 experiments. ** P<0.01.

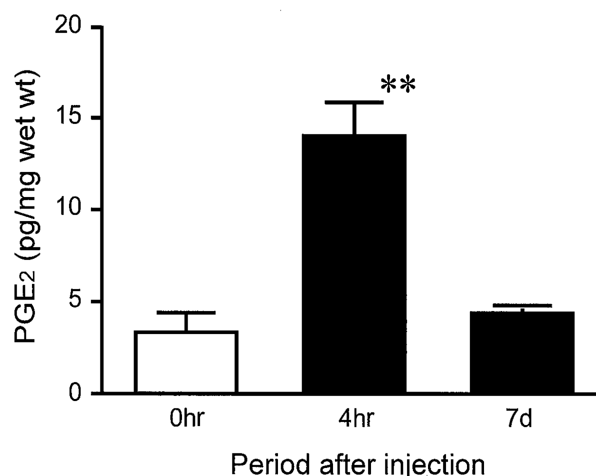

Fig. 3. Amounts of PGE_2 released from ileal smooth muscle strips of rat injected with or without FITC-dextran. At the each period after the injection of dextran, the excised terminal ileum was incubated for 30 min. The incubated supernatant solution (50 μl) was collected for measurements of released PGE_2. Results are means ± SE of mean of 3 experiments. ** P<0.01 vs. 0 hr.

were significantly potentiated by the presence of indomethacin (Fig. 4B).

We next compared the absolute force in the muscles isolated from control and dextran-injected rats. Although the amplitude of contractions due to 65.4 mM KCl in control rat ileum was almost identical to that of contraction in the dextran-injected rat ileum, the absolute force induced by 1 μM carbachol was significantly decreased in dextran-injected rat ileum. In the presence of indomethacin, the carbachol-induced contraction in dextran-injected rat ileum was recovered to almost the same level of contraction observed in control rat ileum (Fig. 4C). Finally, we examined the inhibitory effect of PGE_2 on carbachol (10 μM) and KCl (65.4 mM)-induced contractions in circular smooth muscle isolated from normal rats. Cumulative addition of PGE_2 (0.01-1 μM) more greatly inhibited the carbachol-induced contraction than KCl-induced contraction (Fig. 4D).

Changes in intracellular Ca^{2+} concentration of dextran-phagocytosed resident macrophage: Since intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is one of a critical signal regulating mechanism for the release of inflammatory mediators in macrophages, we measured the changes in $[Ca^{2+}]_i$ in fura-PE3 loaded macrophage to dextran phagocytosis. In this series of experiments, instead of FITC-dextran, we used TexasRed-dextran in order not to interfere the fura-PE3 fluorescence. In freshly isolated macrophage cells, addition of TexasRed-dextran (0.2 mg/ml) into buffer solution induced a gradual increase in $[Ca^{2+}]_i$ which sustained over 30 min (Fig. 5A-C, E). The addition of 4 mM EGTA induced rapid decrease in $[Ca^{2+}]_i$ beyond the resting level. At the end of experiment, we confirmed that macrophages phagocytosed dextran by detecting TexasRed fluorescence (Fig. 5D).

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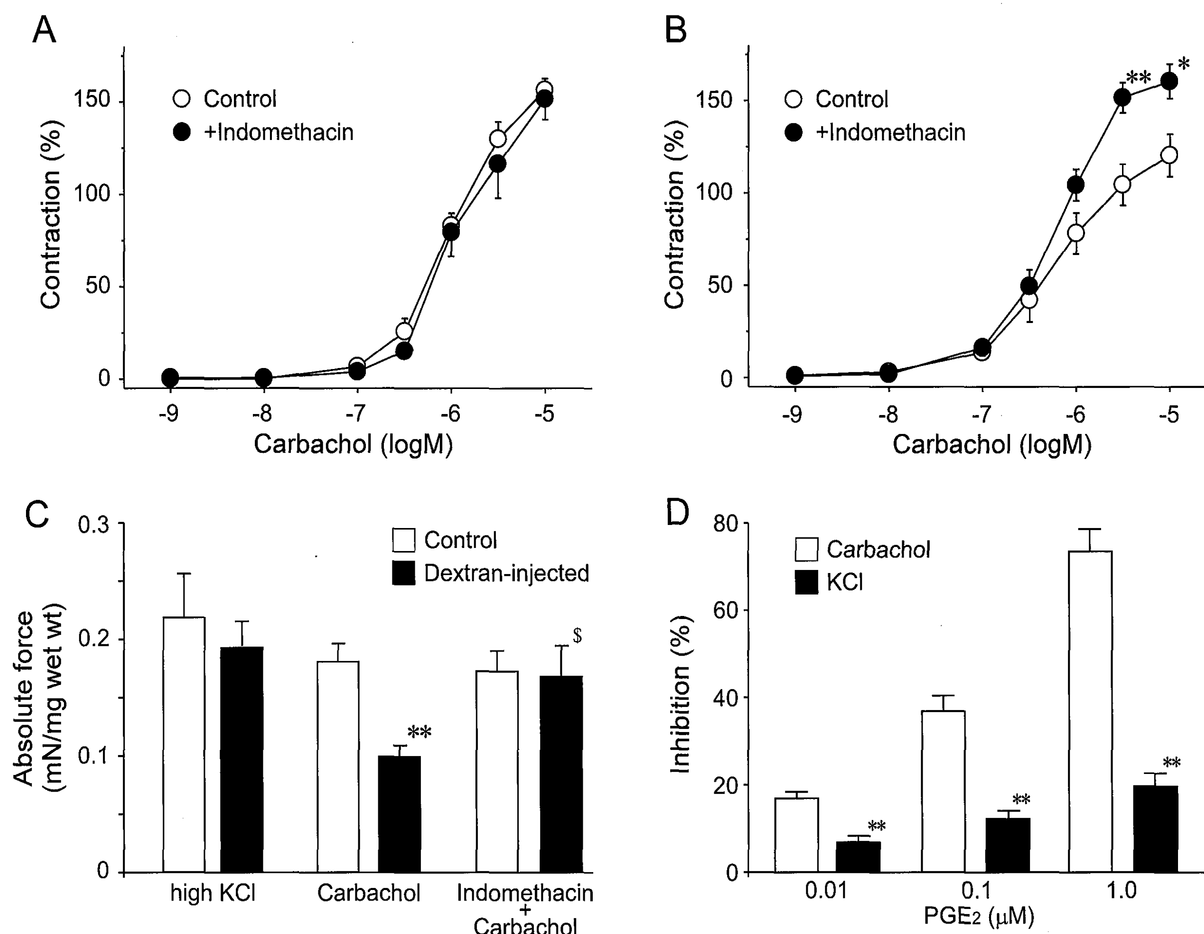

Fig. 4. Suppression of circular smooth muscle contractions in rat ileum after the injection of dextran and its recovery by indomethacin and the inhibitory effect of PGE_2 on carbachol and KCl-induced contractions in normal rat ileum. Rats were injected without (A) or with dextran (B) 4 hr before excising the ileum. Carbachol (1 nM-10 μM) was cumulatively added to the ileal strip to induce contraction in the absence or in the presence of 10 μM indomethacin. Values are expressed as percentage of the reference response (65.4 mM KCl) in each muscle. * $P<0.05$, ** $P<0.01$ vs. contraction in the absence of indomethacin. (C) Absolute force of rat ileum injected without or with dextran induced by 65.4 mM KCl, 1 μM carbachol and 10 μM indomethacin with 1 μM carbachol are compared. ** $P<0.01$ vs. control rat. $ $P<0.05$ vs. carbachol-induced contraction in the absence of indomethacin. (D) Inhibitory effect of PGE_2 on carbachol and KCl-induced contractions in normal rat ileum. PGE_2 (0.01–1 μM) was cumulatively added to the contraction induce by carbachol (10 μM) or KCl (65.4 mM). Contraction induced by carbachol or KCl just before addition of PGE_2 was considered 100 %. ** $P<0.01$ vs. carbachol. All results are means ± SE of mean of 4–8 experiments.

DISCUSSION

The intraperitoneal injection of zymosan induces acute inflammation including the increase in vascular permeability, pain, leukocyte influx, and eicosanoid production by resident peritoneal macrophages [2, 13, 21]. *In vitro* studies have confirmed the ability of macrophages to release large amounts of arachidonic acid and eicosanoids during the stimulation with agonists or phagocytosis. Freshly prepared macrophage isolated from the spleen or peritoneal cavity responded to phorbol ester and Ca^{2+}-ionophore with the synthesis and release of PGE_2 [8, 25]. In the murine bone marrow-derived macrophages, phagocytosable size of latex beads (1.1-μm) stimulated the release of PGE_2, whereas these macrophages did not respond to nonphagocytosable particles (≥ 40-μm) [24]. In the present study, we demonstrated that the injected FITC-dextran was phagocytosed by muscularis resident macrophages in rat small intestine same as previous reports [15, 20, 26]. The confocal microscopic study for FITC-dextran uptake and immunohistochemical study based on the results of double staining of dextran and COX-2 protein suggest that FITC-dextran-positive resident macrophages became immunoreactive to COX-2 antibody (Figs. 1 and 2).

We have reported that the stimulation of rat small intestinal smooth muscle tissue with LPS in organ culture system increased the expression of COX-2 mRNA, in which the expression began to increase at 30 min and reached a maximal level at 60 min [6]. In the present experiments, the numbers of COX-2-immunopositive cells were increased at least at 4 hr after the injection of dextran (Fig. 2), and the COX-2 expression is concurrent with the production of PGE_2 (Fig. 3). These results suggest that the phagocytotic activation of the muscularis resident macrophages is capa-

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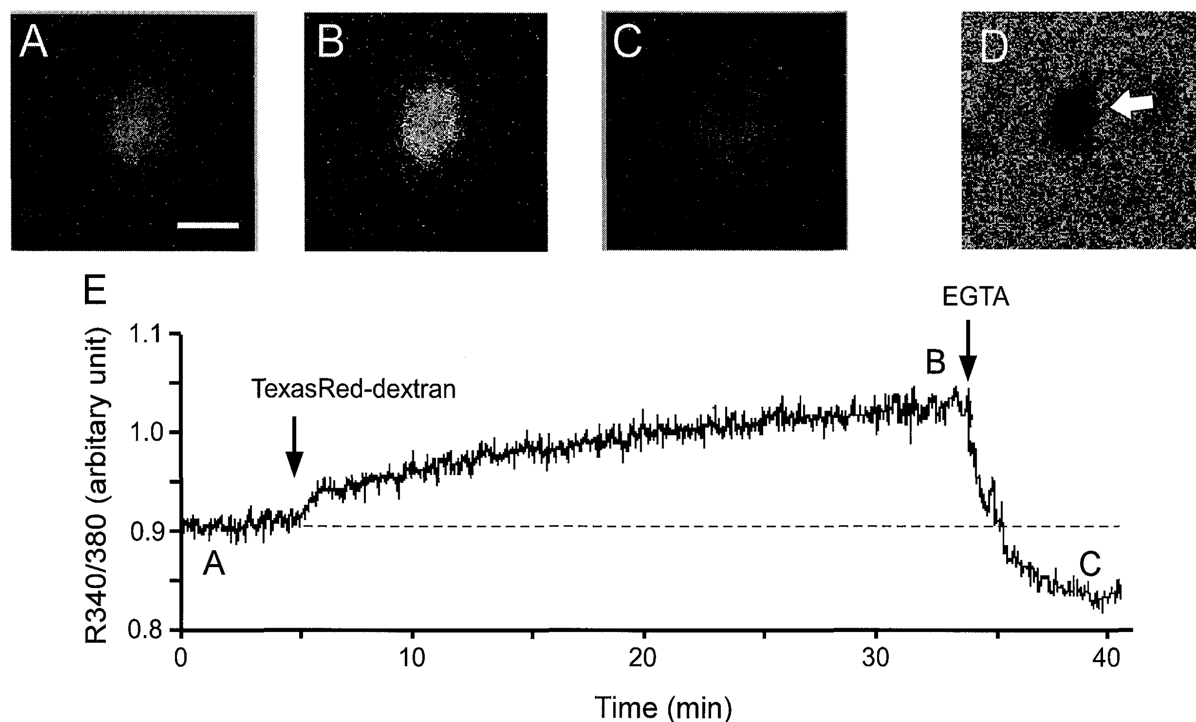

Fig. 5. Effect of TexasRed-dextran (0.2 mg/m*l*) on $[Ca^{2+}]_i$ in a freshly isolated macrophage from control rat ileal smooth muscle layer. TexasRed-dextran produce a gradual increase in $[Ca^{2+}]_i$ as shown by panel E. Calcium images were taken at a time indicated in panel E (Panels A-C). Phagocytosed image of TexasRed-dextran is shown in panel D (Arrow indicates a phagocytosed dextran). Typical results out of 3 experiments are shown. Scale bar, 10 μm.

ble of inducing expression of COX-2 proteins which leads to the production of eicosanoids. The considerable numbers of COX-2 positive cells were still observed after 7 days injection; however, the production of PGE_2 terminated. PGE_2 from arachidonic acid requires at least two enzymes acting sequentially. First, COX catalyses the formation of PGH_2 from arachidonic acid; the second enzyme, PGE_2 synthase converts PGH_2 into PGE_2 [17]. One possible explanation for the dissociation is that PGE_2 synthase has been downregulated in the rat ileum after 7 days injection of dextran. Further study is needed to explain the relationship between COX-2 expression and eicosanoids production at the late phase.

In the animal models for inflammatory bowel disease (IBD) or Hirschsprung's disease, the inflammation of intestinal muscularis is accompanied with the increase in the number of resident macrophage at the level of myenteric plexus layer [10, 26]. In the present results, however, the number of ED2 positive cells was not changed in the dextran injected rat (Fig. 2E). In the inflammatory condition of intestine, a number of cytokines, including TNF-α, IL-6 and IL-1β are released and these mediators stimulate the infiltration of monocytes and activate the macrophages [3]. Distinct from the *in vivo* experimental inflammatory conditions, the stimulation of the macrophage by the phagocytosis seems not to be connected to the processes involving multiplication of macrophage cells.

In the present study, the carbachol-induced contraction of ileal circular smooth muscle was suppressed by dextran-injection (Fig. 4). The suppressed carbachol-induced contraction was recovered almost to the same level as that of control rat by the pretreatment with indomethacin. These results imply that the phagocytosis stimulates the expression of COX-2, which subsequently stimulates the production of inhibitory prostaglandins such as PGE_2, and finally results in the suppression of circular smooth muscle contraction. On the other hand, the 65.4 mM KCl-induced contraction was not suppressed in the dextran-injected rat ileum. It was reported that the relaxation of bethanechol-stimulated contraction by PGE_2 is associated with the increased production of intracellular cAMP in intestinal circular smooth muscle. It is known that the receptor agonist-induced contraction is more sensitive to cAMP-dependent relaxation system than depolarization-induced contraction [9, 19]. Therefore, we next examined the direct effect of PGE_2 on carbachol and KCl-induced contractions. We found that the addition of PGE_2 vigorously inhibited carbachol-induced contraction than KCl-induced contraction (Fig. 4D).

It is widely accepted that intracellular Ca^{2+} regulates various cellular responses in macrophages, including proliferation, cell growth, migration, cell adhesion, apoptosis, gene transcription and the release of proinflammatory mediators such as superoxides, cytokines and arachidonic acid. Macrophages express three classes of phospholipase A_2 (PLA_2) and two of these are activated in a Ca^{2+}-dependent manner [1]. Several agonists have been shown to elicit an increase

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in $[Ca^{2+}]_i$ in macrophages [7, 12, 31]. In mouse intestinal resident macrophages, we have already shown that agonists such as ATP, PAF and LPS increased $[Ca^{2+}]_i$ [20]. In the present study, we demonstrated that $[Ca^{2+}]_i$ was increased in the rat muscularis macrophages in the presence of dextran, indicating that foreign body outside the macrophage functions as the Ca^{2+} signal agonist. This response occurred slowly and was long lasting (Fig. 5).

In summary, our study demonstrates that phagocytosis of dextran by the resident macrophages in the rat small intestinal muscularis layer stimulates COX-2 gene expression, and its product PGE$_2$ reduced muscle contractility. Resident macrophages in the muscle layer play a key role in gastrointestinal motility under pathological conditions.

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