Role of Constitutive Cyclooxygenase-2 in Prostaglandin-Dependent Secretion in Mouse Colon In Vitro

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Abstract

The relative contributions of cyclooxygenase (COX)-1 and COX-2 in mediating prostaglandin (PG)-dependent chloride secretion were investigated in segments of mouse colon mounted in Ussing-type diffusion chambers. COX-2 mRNA and protein were constitutively expressed as shown by reverse transcription-polymerase chain reaction and Western immunoblot, respectively. COX-2 immunoreactivity was detected immunohistochemically in cells lying subjacent to the crypt epithelial cells. In segments of colon mounted in Ussing chambers, arachidonic acid caused a concentration-dependent increase in short-circuit current that was blocked by piroxicam, the COX-2 inhibitor NS-398, and the COX-1 inhibitor SC-560. Exposure to the PG-dependent secretagogue, bradykinin, also caused an increase in short-circuit current that was not blocked by piroxicam or SC-560, and only by the highest dose of NS-398. When incubated in the presence of 10 μM arachidonic acid, segments of mouse colon produced both PGE₂ and PGD₂. Synthesis of PGE₂ but not PGD₂ was blocked by NS-398 and SC-560. These data demonstrate that both COX-1 and COX-2 are constitutively expressed in the mouse colon, and both contribute to PG-dependent electrolyte transport.

Prostaglandins (PGs) are produced from free arachidonic acid through the catalytic activity of two cyclooxygenase (COX) enzymes. Until recently, COX-1 was considered the constitutive isoform, being expressed in almost all tissues, whereas COX-2 was considered inducible, because its expression could be triggered by inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α. This paradigm led to the belief that COX-1, which is found in abundance in the gastrointestinal tract, produced PGs that were cytoprotective and that subserved physiological functions such as control of mucosal blood flow and electrolyte secretion. In contrast, COX-2-derived PGs were considered deleterious because they were found at sites of inflammation (where COX-2 was induced) and were hence thought to trigger, participate in, or exacerbate the inflammatory response. This dogma has provided the impetus for the recent development of selective COX-2 inhibitors that would inhibit inflammatory COX-2 and spare cytoprotective COX-1 (Masferrer et al., 1994; Hawkey, 1999). Traditional nonsteroidal anti-inflammatory drugs are relatively nonselective and their chronic use in conditions such as rheumatoid arthritis is associated with a high incidence of serious adverse gastrointestinal events (Wallace and Granger, 1992).

Recently, however, evidence has mounted that clouds the “COX-1 is good, COX-2 is bad” paradigm. Several groups have reported constitutive expression of COX-2 in numerous tissues, including the kidney (Harris et al., 1999), female reproductive tract (Slater et al., 1994) and fetal membranes (Slater et al., 1999), central nervous system (Yasojima et al., 1999), and vascular endothelium (McAdam et al., 1999). We have shown in a carrageenan-induced rat paw edema model that COX-2 inhibitors only exhibited anti-inflammatory effects at concentrations that have been shown to also inhibit COX-1 (Wallace et al., 1998). In addition, it has recently been shown that COX-2-derived PGs may exhibit anti-inflammatory properties (Gilroy et al., 1999). A further complicating observation is the demonstration that COX-1 can be induced under certain circumstances (Jun et al., 1999). Given these recent data, it is clear that the physiological roles of the cyclooxygenases need to be reevaluated.

In the intestinal tract, secretion of chloride ions and water into the lumen is considered, along with low epithelial permeability, part of a primary host defense mechanism preventing the translocation of luminal bacteria, bacterial products, and antigens into the mucosa (Wood, 1993). Permeability defects have been implicated in relapse of inflammatory bowel disease (Wyatt et al., 1993) and secretory...
function is suppressed in inflamed intestine (Goldhill et al., 1993; MacNaughton et al., 1998). It has long been known that the response of the epithelium to numerous secretagogues is regulated by PGs. PGE₂, probably derived from subepithelial myofibroblasts, is a "common final mediator" of secretagogues encompassing various lipid mediators, cytokines, oxygen radicals, amines, and enzymes (Hinterleitner and Powell, 1991). It acts both directly on the enterocyte and via submucosal secretomotor neurons (Dekkers et al., 1997).

In contrast, PGD₂ is antisercretoery, functioning to block secretion stimulated by submucosal secretomotor neurons of the enteric nervous system (Goerg et al., 1992). The balance between PGE₂ and PGD₂ is important in the regulation of epithelial electrolyte transport (Keenan and Rangachari, 1989). However, the COX isoform responsible for production of PGs involved in intestinal secretion is not known. Given the role of PGs in regulating this important host defense mechanism, and the increasing interest in selective COX-2 inhibitors, we sought to determine the relative roles of COX-1- and COX-2-derived PGs in mediating PG-dependent chloride secretion in a mouse model.

Experimental Procedures

Animals

Male C57Bl/6 mice (4–6 weeks of age) were obtained from Charles River (Montreal, Quebec, Canada) and were housed in wire-bottomed cages under constant temperature (22°C) and photoperiod (12-h light/dark cycle). They were allowed unrestricted access to standard mouse chow and water. Mice were allowed to acclimatize to these conditions for at least 5 days before inclusion in an experiment. All procedures involving animals were approved by the University of Calgary Animal Care Committee and were conducted according to the guidelines of the Canadian Council on Animal Care.

COX-1 and COX-2 Expression and Localization

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Mice were euthanized by cervical dislocation. Colonos were removed and rinsed in PBS consisting of 137 mM NaCl, 8.16 mM Na₂HPO₄, 1.47 mM KH₂PO₄, and 3.22 mM KCl. Samples were homogenized in 1 ml of suspension buffer (0.1 M NaCl; 0.01 M Tris-HCl, pH 7.6; 0.001 M EDTA, pH 8.0; 1 µg/ml aprotinin; 100 µg/ml phenylmethylsulfonyl fluoride; and 1% Triton X-100) and centrifuged at 12,000 g for 5 min at room temperature. Half of the supernatant was removed and added to an equal volume of sample buffer (catalog no. S-3401; Sigma Chemical Co., Mississauga, Ontario, Canada). The other half of the sample was used to determine protein concentration with a protein assay kit (Bio-Rad). Samples were boiled for 10 min and the DNA was sheared by sonication. Samples were then centrifuged at 12,000g for 5 min at room temperature and the supernatant run on a 1% agarose gel containing ethidium bromide. After separation the bands were visualized under UV light with a Gel Doc 2000 (Bio-Rad, Mississauga, Ontario, Canada), and analyzed with Quantity One software (Bio-Rad).

Western Immunoblot. Mice were euthanized by cervical dislocation. Colonos were removed and rinsed in PBS consisting of 137 mM NaCl, 8.16 mM Na₂HPO₄, 1.47 mM KH₂PO₄, and 3.22 mM KCl. Samples were homogenized in 1 ml of suspension buffer (0.1 M NaCl; 0.01 M Tris-HCl, pH 7.6; 0.001 M EDTA, pH 8.0; 1 µg/ml aprotinin; 100 µg/ml phenylmethylsulfonyl fluoride; and 1% Triton X-100) and centrifuged at 12,000g for 5 min at room temperature. Half of the supernatant was removed and added to an equal volume of sample buffer (catalog no. S-3401; Sigma Chemical Co., Mississauga, Ontario, Canada). The other half of the sample was used to determine protein concentration with a protein assay kit (Bio-Rad). Samples were boiled for 10 min and the DNA was sheared by sonication. Samples were then centrifuged at 12,000g for 5 min at room temperature and the supernatant run on an 8% acrylamide gel in a mini trans-blot electrophoretic transfer cell (Bio-Rad). Protein was transferred to nitrocellulose overnight at 30 V and 4°C. After transfer, the membrane was blocked in 5% milk for 1 h and washed briefly in PBS-Tween. The COX-1 or COX-2 primary antibodies (antig- mouse antibodies; Santa Cruz Biotechnologies, Santa Cruz, CA) were diluted 1:75 in 1% milk/PBS-Tween. The membrane was placed in the primary antibody for 1.5 h, and then washed five times in PBS-Tween. The secondary antibody (rabbit anti-goat IgG conjugated to horseradish peroxidase; Santa Cruz Biotechnologies) was diluted 1:10,000 in 1% milk/PBS-Tween. The membrane was placed in the secondary antibody for 1 h, and then washed five times in PBS-Tween. Luminol reagent (Santa Cruz Biotechnologies) was applied to the membrane for 2 min. The membrane was then exposed to X-ray film to visualize the proteins. The X-ray film was analyzed with a GS-710 calibrated imaging densitometer (Bio-Rad) and Quantity One software (Bio-Rad).

Immunohistochemistry. Segments of mouse colon were removed and fixed in Zamboni’s fixative overnight. They were then washed in PBS (three times) and then cryoprotected in PBS containing 20% (w/v) sucrose. Sections (12–14 µm) were cut with a cryostat (Microm GmbH, Heidelberg, Germany) and placed onto poly(t-lysine)-coated microscope slides. Sections were washed three times in PBS and were incubated with a rabbit anti-COX-2 primary antibody at 4°C overnight. Sections were then rinsed with PBS and incubated with donkey anti-rabbit IgG CY3-conjugated secondary antibody for 90 min. They were then washed three times in PBS, coverslipped, and viewed under epifluorescence with a Zeiss Axiosplan microscope. Images were captured and digitized with a charge-coupled device video camera and Northern Exposure imaging software (Carse Vision, Edmonton, Alberta, Canada).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sequences of primers for PCR</th>
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<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>COX-1</td>
<td>5'-CCAACGTTGATTTACACTCG</td>
</tr>
<tr>
<td>COX-2</td>
<td>3'-GGTTGAGGTTGTTGAACTCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CAGAGTACACTACATGACC</td>
</tr>
<tr>
<td>3'-AGCTTTCTCCACGCTGCTTAT</td>
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μl of 10× PCR buffer, and 35 μl of double-distilled water. Two microliters of each of the 5′ and 3′ primers (Table 1) were added for COX-1 and COX-2 and GAPDH. GAPDH was used as an internal control. Taq polymerase was added with a hot start to reduce nonspecific binding. The samples were cycled as follows: 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min. Optimal amplification was achieved at 32 cycles for COX-1 and COX-2 and 22 cycles for GAPDH. The PCR products were run on a 1% agarose gel containing ethidium bromide. After separation the bands were visualized under UV light with a Gel Doc 2000 (Bio-Rad, Mississauga, Ontario, Canada), and analyzed with Quantity One software (Bio-Rad).
Mice were sacrificed by cervical dislocation and segments of colon removed and gently flushed with cold Krebs’ buffer (4°C). The segments were opened longitudinally along the mesenteric border and mounted on standard Ussing-type diffusion chambers (Navicyte Inc., Sparks, NV). Tissues were bathed on both the mucosal and serosal surfaces with Krebs’ buffer containing 10 mM d-glucose. The bathing solutions were maintained at 37°C and pH 7.4 and were aerated with 5% CO₂, 95% O₂. The transepithelial potential difference was maintained at zero volts by applying a short-circuit current (Iₑ) with a voltage clamp apparatus (EVC4000; World Precision Instruments, Sarasota, FL). The Iₑ was taken as the measure of net active electrolyte transport by the colonic epithelium. Tissues were allowed to equilibrate with respect to Iₑ for 15 to 20 min. Drugs were applied to the serosal bath.

Materials

Routine chemicals were obtained from BDH Chemicals (Toronto, Ontario, Canada). NS-398 was obtained from Research Biochemicals Inc. (Natick, MA). SC-560 was a generous donation from Dr. Frank Degner, Boehringer-Ingelheim GmbH. Piroxicam was obtained from Sigma Chemical Co. Other chemicals were obtained as indicated in the text.

Statistical Analysis

Data are expressed as the mean ± SE. Comparisons among groups were made by one-way ANOVA with post hoc Newman-Kuels test with Instat version 3.00 (GraphPad Software, San Diego, CA). A P value of <.05 was considered to be significant.

Results

Expression of COX-2 in Mouse Colon. COX-1 and COX-2 mRNA expression was observed in full-thickness segments of mouse colon (n = 3), as demonstrated by RT-PCR (Fig. 1). Western blot analysis revealed COX-1 and COX-2 proteins in full-thickness segments of mouse colon (n = 3; Fig. 1).

COX-2 immunoreactivity (COX-2-IR) in normal mouse colon is shown in Fig. 2. COX-2-IR was not observed in the epithelium but was evident in cells subjacent to the epithelium. This microanatomical localization is consistent with, but not proof of, identification of these cells as myofibroblasts. Furthermore, COX-2-IR was observed in the muscularis externa, particularly in the longitudinal muscle layer. Routine histology indicated that there was no ongoing chronic inflammation; there was no evidence of an inflammatory cell infiltrate, mucosal or submucosal edema, or muscle thickening (data not shown). Clinically, the animals exhibited no symptoms such as diarrhea.

Prostaglandin-Dependent Secretion. Concentration-response relationships were established for the PG-dependent secretagogues bradykinin (1 nM–1 μM; Musch et al., 1983) and arachidonic acid (1–100 mM; Field et al., 1984). Both caused concentration-dependent increases in Iₑ in unstripped segments of mouse colon mounted in Ussing chambers (Fig. 3). The response to arachidonic acid occurred within 1 min, peaked within 5 min, and either remained elevated or returned to baseline only slowly. The Iₑ remained elevated above baseline for the duration of the experiment.

Fig. 1. Constitutive expression of COX-1 and COX-2 in mouse colon. A, results (reversed image) of RT-PCR assay for COX-1 (left) and COX-2 (right) mRNA expression in segments of colon from three C57BL/6 mice. B, COX-1 (left) and COX-2 (right) protein expression as shown by Western immunoblot. Positive immunoreactivity was seen in segments of colon from three mice.

Fig. 2. COX-2 immunoreactivity in mouse colon. A, full thickness cross-section of mouse colon. Mucosal staining was only observed in subepithelial cells (arrowheads) at the bases of the crypts (Cr). Positive staining also was observed in the longitudinal muscle layer. B, section of mouse colon through mucosal crypts (Cr). Staining in subepithelial cells is observed (arrowheads). Scale bar, 50 μm.

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The response to bradykinin also began within 1 min, peaked within 3 min, and then returned to baseline over the next 5 min.

The effect of selective inhibitors of COX-1 and COX-2 on the responses to arachidonic acid and bradykinin were examined in vitro. Doses of the selective COX-2 inhibitor NS-398 and the selective COX-1 inhibitor SC-560 were chosen based on published pharmacological data demonstrating their selectivity in in vitro systems (Futaki et al., 1994; Smith et al., 1998). A high dose (100 μM) of the nonselective inhibitor piroxicam was used to block both COX-1 and COX-2. The selective COX-2 inhibitor NS-398 caused a concentration-dependent inhibition of the response to arachidonic acid that was almost complete at 30 μM (Figs. 4 and 5). The secretory response to arachidonic acid also was blocked by the selective COX-1 inhibitor SC-560 at nanomolar concentrations and by the nonselective COX inhibitor piroxicam (Fig. 5).

Interestingly, the response to 10 nM bradykinin was not blocked by piroxicam (100 μM), NS-398 (1–3 μM), or SC-560 (0.1–1 μM). Only NS-398 at 30 μM resulted in a significant reduction in the response to bradykinin (Fig. 5).

Prostaglandin Synthesis. To determine the degree to which the COX inhibitors blocked PG synthesis, further experiments were conducted in which whole-thickness segments of mouse colon were incubated at 37°C in Krebs buffer containing 10 μM arachidonic acid or 10 nM bradykinin plus piroxicam (100 μM), NS-398 (1–30 μM), or SC-560 (0.1–1 μM). These concentrations of arachidonic acid and bradykinin stimulated chloride secretion in Ussing chamber studies (see above). Controls were incubated with vehicle, arachidonic acid, or bradykinin only. After 10 min, the tissues were removed and processed for measurement of PGs as previously described (Wallace et al., 1990). Tissue levels of PGE₂ and PGD₂ were determined with commercial enzyme-linked immunosorbent assay kits (Cayman Chemical, Ann Arbor, MI). The levels of PG synthesis in the presence of the inhibitors were expressed as a percentage of the maximal response, in the presence of arachidonic acid or bradykinin, to take interassay variation into account. In the presence of arachidonic acid, piroxicam almost completely blocked PGE₂ synthesis (Fig. 6). NS-398 caused a concentration-dependent inhibition of PGE₂ that was almost complete at 30 μM. SC-560 also reduced PGE₂ synthesis in a dose-dependent manner, but did not cause more than an ~55% inhibition (Fig. 6). In the same tissues, PGD₂ synthesis was significantly inhibited by 100 μM piroxicam but not by NS-398 (1–30 μM) or SC-560 (0.1–1 μM; Fig. 6). Bradykinin did not stimulate PGE₂ or PGD₂ synthesis compared with unstimulated controls (Fig. 7). NS-398 (30 μM) and SC-560 (300 nM) reduced PGE₂ synthesis in the presence of bradykinin. Neither inhibitor significantly affected PGD₂ synthesis. Piroxicam induced a significant reduction in PG synthesis compared with controls (Fig. 7).
the PG synthesis observed without addition of inhibitors. Top, PGE$_2$ synthesis; bottom, PGD$_2$ synthesis. **P < .01, ***P < .001 versus control.

Fig. 6. PG synthesis in isolated segments of mouse colon incubated with 10 µM arachidonic acid for 10 min. Data are expressed as a percentage of the PG synthesis observed without addition of inhibitors. Top, PGE$_2$ synthesis; bottom, PGD$_2$ synthesis. **P < .01, ***P < .001 versus control.

Fig. 7. PG synthesis in isolated segments of mouse colon incubated with 10 nM bradykinin for 10 min. Data are expressed as a percentage of the PG synthesis observed without addition of bradykinin. ■, PGE$_2$; □, PGD$_2$. *P < .05, ***P < .001.

Discussion

In this study, we have demonstrated the constitutive expression of both COX-1 and COX-2 in the mouse colon, and have provided evidence that both COX-1 and COX-2 contribute to PG-dependent secretion in vitro. It has now been widely demonstrated that COX-2 is constitutively expressed in a variety of tissues, including brain, kidney, female reproductive tract, fetal tissues, and vascular endothelium. As expected, COX-1 was constitutively expressed in mouse colon. However, we now demonstrate constitutive COX-2 expression in mouse colon. In each mouse studied, COX-2 mRNA and protein were expressed. COX-2 expression was not due to ongoing inflammation because none of the mice exhibited clinical, macroscopic, or microscopic signs of colonic inflammation. Of note, we also have shown constitutive co-

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lionic expression of the inducible isofrom of nitric oxide synthase in the absence of inflammation (MacNaughton et al., 1998). Based on our immunohistochemical data, it appears that COX-2 is expressed primarily in cells lying subjacent to the crypt enterocytes. The subepithelial myofibroblasts have long been known to be a source of PGs that regulate epithelial function (Hinterleitner and Powell, 1991) and are localized to this region of the mucosa. However, the isofrom of COX present in these cells, and which is responsible for the production of secretory PGE$_2$, is not known. Our data are suggestive of, but not proof of, subepithelial myofibroblasts as the cellular source of COX-2-derived PGs regulating epithelial secretory function.

Both arachidonic acid and bradykinin caused concentration-dependent increases in short-circuit current in segments of mouse colon mounted in Ussing chambers. Both of these secretagogues have been reported to stimulate chloride secretion through the synthesis of PGs (Musch et al., 1983; Field et al., 1984). Pretreatment of the tissue with the nonselective COX inhibitor piroxicam almost abolished the response to arachidonic acid, but failed to significantly inhibit the response to bradykinin. The response to arachidonic acid was significantly and concentration-dependently blocked by the COX-2 inhibitor NS-398, suggesting that a substantial portion of arachidonic acid-mediated secretion was through a COX-2-dependent mechanism. Similarly, the response to arachidonic acid was reduced by pretreatment with the selective COX-1 inhibitor SC-560. That the response could be blocked by both selective COX-1 and COX-2 inhibitors was curious. The most obvious explanation would be that one or both of these inhibitors were acting nonselectively. However, care was taken to use concentrations of NS-398 and SC-560 that have previously been shown to be selective for COX-2 and COX-1, respectively (Futaki et al., 1994; Smith et al., 1998). NS-398 at 100 µM did not block the activity of COX-1 isolated from sheep seminal vesicles, whereas the IC$_{50}$ of NS-398 against COX-2 isolated from sheep placenta was 3.8 µM (Futaki et al., 1994). Although action against isolated, purified enzyme may not equate to activity in tissue, 30 µM NS-398, the highest concentration used herein, has been reported to be a selective COX-2 inhibitor in rat aortic organ culture (Hamilton et al., 1999). SC-560 demonstrated a 1000-fold higher selectivity for human recombinant COX-1 than for human recombinant COX-2 in vitro (Smith et al., 1998).

The lack of effect of COX inhibition on the secretory response to bradykinin was unexpected, given the previous reports of the PG-dependence of this response (Musch et al., 1983; Field et al., 1984). A possible explanation may be that bradykinin was added to the tissue in the presence of arachidonic acid. The availability of arachidonic acid has been shown to significantly affect the ability of COX-2 inhibitors to block COX-2 (Hamilton et al., 1999). In our study, only the highest dose of NS-398 (30 µM) significantly blocked the response to bradykinin. However, the ability of the COX inhibitors to block the response to arachidonic acid itself in these preparations argues against an effect of arachidonic acid on their ability to block the subsequent response to bradykinin. It may be that in the mouse colon, there is a substantial PG-independent component to the secretory response, and this is confirmed by the experiments showing a lack of effect of bradykinin on PG synthesis, at a concentration that elicited a change in $I_{sc}$. Indeed, it has been reported...
that there is a substantial neural component to the response to bradykinin in rat (Perkins et al., 1988) and dog (Rangachari et al., 1993) colons. Our data support a PG-independent response to bradykinin in mouse colon, however, determining the exact mechanism of action was beyond the scope of this study.

To further characterize the isoforms of COX present in the mouse colon, experiments were conducted in which isolated, full-thickness segments of colon were incubated in the presence of arachidonic acid or bradykinin and either NS-398, SC-560, or piroxicam, with subsequent measurement of tissue levels of PGE$_2$ and PGD$_2$. Arachidonic acid induced PG synthesis. NS-398 caused a concentration-dependent decrease in the synthesis of PGE$_2$, with almost complete inhibition at 30 μM. Similarly, piroxicam at 100 μM abolished PGE$_2$ synthesis. SC-560 only reduced PGE$_2$ synthesis to ~55% of control. This is interesting in that these same doses of SC-560 almost completely abolished arachidonic acid-induced chloride secretion. These data suggest that there may be a PG-independent inhibitory effect of SC-560 on arachidonic acid-induced chloride secretion in this in vitro model. Interestingly, bradykinin, at a dose that stimulated chloride secretion, did not stimulate PG synthesis. This supports our contention (see above) that bradykinin-induced secretion in the mouse colon is dependent on mechanisms other than PGE$_2$, and represents a significant species difference compared with guinea pig and rabbit.

Although NS-398 and SC-560 inhibited PGE$_2$ synthesis, they did not significantly block the synthesis of PGD$_2$. The synthesis of PGD$_2$ was, however, almost completely blocked by 100 μM piroxicam. The differential effect of the COX inhibitors on PGE$_2$ and PGD$_2$ synthesis was not expected because both COX-1 and COX-2 catalyze the production of PGH$_2$, the precursor for both PGE$_2$ and PGD$_2$. The results suggest that the effect of NS-398 in particular may be a combination of inhibition of COX-2 and a further downstream step in the synthesis of PGE$_2$ from PGH$_2$. However, NS-398 did not affect the chloride secretory response to serosally applied PGE$_2$ (data not shown) argues against this conclusion. Further studies will be required to determine the exact mechanism of action in this regard. That these compounds can block PGE$_2$ synthesis, although leaving PGD$_2$ synthesis relatively untouched, has important physiological implications for mucosal function. PGE$_2$ and PGD$_2$ have important clinical implications for mucosal function. PGE$_2$ and PGD$_2$ have important clinical implications for mucosal function.

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**References**


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