The FASEB Journal Express article fj.05-5518fje. Published online July 28, 2006.

Effects of sphingosine-1-phosphate and ceramide-1-phosphate on rat intestinal smooth muscle cells: implications for postoperative ileus

Mihaela Dragusin, Sven Wehner,* Samuel Kelly,† Elaine Wang,† Alfred H. Merrill, Jr.,† Jörg C. Kalff,* and Gerhild van Echten-Deckert[1]

Kekulé-Institute for Organic Chemistry and Biochemistry, University Bonn, Germany; *Department of Surgery, University Bonn, Germany; and †School of Biology and Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia, USA

ABSTRACT **Postoperative ileus, a major cause of morbidity after abdominal surgery, is characterized by intestinal dysmotility and inflammation. The aim was to investigate the involvement of sphingolipids in postoperative intestinal inflammation using a standardized rat model of intestinal surgical manipulation. Sphingolipid analysis (ESI-MS) of intestinal muscularis after manipulation revealed a time-dependent increase of sphingosine 1-phosphate (S1P) and of ceramide 1-phosphate (C1P). We therefore established a culture system of primary rat intestinal smooth muscle cells and examined the potential role of these sphingolipids in intestinal inflammation. Incubation of cells with either of the two sphingolipid-phosphates resulted in an elevated production of PGE_2. Further analysis revealed that S1P enhances cyclooxygenase 2 (COX-2) expression whereas C1P increases release of arachidonic acid, indicating an enhanced phospholipase A_2 activity. S1P-induced COX-2 expression was pertussis toxin sensitive, suggesting the involvement of Gi/o protein-coupled S1P receptors. Further downstream mediators of S1P induced COX-2 expression appear to be extracellular regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK). Collectively, our results demonstrate that intestinal smooth muscle cells represent a major target for both C1P and S1P activity. Thus, the sustained elevated concentration of the two bioactive sphingolipids in this tissue could at least in part explain postoperative intestinal dysmotility.—Dragusin, M., Wehner, S., Kelly, S., Wang, E., Merrill, A. H., Jr., Kalff, J. C., van Echten-Deckert, G. Effects of sphingosine-1-phosphate and ceramide-1-phosphate on rat intestinal smooth muscle cells: implications for postoperative ileus. *FASEB J.* 20, 000–000 (2006)**

Key Words: sphingolipids · inflammation · COX-2 · PGE_2

Postoperative ileus or "paralysis of the bowel" is a life-threatening impairment of bowel motility that occurs after surgery. The increased medical costs resulting from prolonged hospitalization were estimated as nearly $1 billion annually in the U.S. (1). Several pathogenic mechanisms responsible for postoperative dysmotility have been proposed including autonomic nervous system, release of inhibitory humoral agents, the influence of anesthetics, as well as local factors including inflammation (1). Inflammatory response is regulated by numerous cell surface receptors and ligands along with the release of chemokines, cytokines, vasoactive amines and NO. However, mechanistic studies of inflammation progressively revealed a key role of bioactive eicosanoids and sphingolipids in inflammatory responses (2). Eicosanoids, including prostaglandins (PGs), are generated from arachidonic acid (AA), a polyunsaturated fatty acid (C20: 4) released from cell membrane phospholipids by the action of the rate-limiting enzyme phospholipase A_2 (PLA_2). AA is then converted to PGs via the cycooxygenase (COX) pathway. Of the two known COX isoforms, COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced predominantly during acute or chronic inflammation (3). Sphingolipids (SLs) are a novel class of bioactive lipids that play key roles in the regulation of several cellular processes including growth, differentiation, stress response, and apoptosis (4). Earlier studies revealed that sphingosine and ceramide, two SLs known to act as negative regulators of cell proliferation, are able to induce production of the inflammatory mediator PGE_2 (5, 6). More recent studies have implicated a regulatory function of S1P and C1P, the phosphorylated forms of sphingosine and ceramide, respectively, in PGE_2 production (7, 8). Whereas S1P has been shown to trigger COX-2 induction, C1P appears to be involved in translocation and thus activation of cytosolic PLA_2 (9, 10).

It has been shown that simple mechanical contact of the gut during abdominal surgery elicits an inflammatory cascade in the intestinal *tunica muscularis externa* accompanied by suppression of circular smooth muscle

[1]Correspondence: Kekulé-Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Strasse 1, Bonn, 53121 Germany. E-mail: g.echten.deckert@uni-bonn.de
doi: 10.1096/fj.05-5518fje

contractility, and hence decreased intestinal transit (11). In addition, it was shown recently that intestinal surgical stress is accompanied by induction of the proinflammatory cytokine interleukin (IL) -6 within the *tunica muscularis externa* of rodent small bowel (12). Moreover, the active role of smooth muscle cells in the manifestation of inflammatory responses has been documented (13, 14). Thus, primary cultured intestinal longitudinal muscle cells were reported to secrete cytokine IL-6 in response to IL-1β (13). Also, colonic smooth muscle cells were suggested to synthesize a broad range of mediators and signaling proteins that might contribute to inflammation (14).

In the present study we investigated the involvement of sphingolipids in postoperative ileus using a standardized surgical rodent model of intestinal manipulation. The finding that "gentle" surgical manipulation of the small bowel results in an increase of S1P and of C1P amounts only in the intestinal *tunica muscularis externa* prompted us to analyze the potential involvement of these two SL-phosphates in inflammatory processes in primary cultured rat intestinal smooth muscle cells. Our studies prove that C1P and S1P enhance the production of PGE_2 in these cells, probably by contributing to the modulation of the enzymatic activity of PLA_2 and COX-2, respectively.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (200–250 g) were obtained from Harlan Winkelman, (Borchen, Germany). SB239063 was kindly provided by GlaxoSmithKline. S1P, phosphatidic acid (1,2 didecanoyl-sn-glycerol 3-phosphate), PD98059, SP600125, Accuspin, HBSS, and X-ray films were purchased from Sigma (Taufkirchen, Germany). [1- (14) C] arachidonic acid (56mCi/mmol) was from Amersham-Buchler (Braunschweig, Germany) and C8-C1P was from Biomol (Hamburg, Germany). Dulbecco's modified Eagle medium (DMEM) -F12 (DMEM-F12), Minimal essential medium, trypsin, FBS, BSA, and goat antimouse IgG labeled with green fluorescent Alexa Fluor 488 dye were obtained from Invitrogen (Karlsruhe, Germany). Antibodies recognizing the phosphorylated forms of ERK and p38 MAPK, and both phosphorylated and unphosphorylated forms of ERK and p38 MAPK were from NEB (Frankfurt, Germany), while antibodies against COX-2 and α-tubulin were from Santa Cruz Biotechnology (Heidelberg, Germany). Primary antibody (Ab) against smooth muscle α-actin was obtained from Dianova (Dörentrup, Germany). Prostaglandin E_2 enzyme immunoassay (EIA) kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). Ceramide and all internal standards for the sphingolipid analyses were from Avanti Polar Lipids (Alabaster, AL, USA).

Experimental groups and operative procedures

The small bowel of the animals was subjected to standardized, nontraumatic intestinal manipulation, as described (15). In brief, the animals were anesthetized by inhalation anesthesia, and a midline incision was made into the peritoneal cavity. The small bowel was eviscerated to the left onto a mostly cotton gauze pad, and the entire small bowel was lightly manipulated with moist cotton applicators. The operative procedures were performed under sterile conditions. Nonoperated animals served as controls. In the time course study, three groups of five animals were evaluated, each at a different time point (3, 6, 12, 24 h) after intestinal manipulation. In all groups, the operative time was similar and the midline incision was closed by two-layer continuous sutures. The animals recovered from the procedure within 10 min. Small bowel preparations from these animals were used for sphingolipid extraction.

Small bowel preparation

Animals were anesthetized at sacrifice and small bowel specimens were harvested as described previously (15). In brief, the abdominal wound was reopened and the abdominal aorta cannulated and flushed with 3 ml of cold (4°C) Ringer solution (Baxter, Deerfield, IL, USA). This flush was used to remove nonadherent and nonextravasated blood cells from the muscularis vasculature. The entire small bowel was then removed and placed in cold preoxygenated Krebs-Ringer buffer. The small bowel was used for the selective isolation of the muscularis as described previously (16). The isolated layers were frozen in liquid nitrogen.

Isolation of cultured rodent intestinal smooth muscle cells

The muscularis tissue was isolated, as described above, cut into small pieces, washed twice in HBSS and incubated with 0,6 mg/ml type II collagenase (Worthington Biochemical Corp., Freehold, NJ, USA) and 2 U/ml dispase II (Hoffmann-LaRoche, Nutley, NJ, USA) in HBSS for 45 min at 37°C in a shaking water bath. Muscle cells dispersed from the muscularis layer were harvested by filtration using a 500 μm Nitex mesh and centrifuged at 300 *g* for 5 min. Cells were resuspended and washed twice by centrifugation at 300 *g* for 5 min. After resuspension, 3 ml of accuspin was added carefully prior to density gradient centrifugation (20 min, 800 *g*, 4°C). Two phases and a pellet containing muscle cells were obtained. The pellet was resuspended in DMEM-F12 containing 10% FBS and 1% penicillin/streptomycin. Cultures were incubated in a 5% CO_2 environment at 37°C. DMEM-F12 medium was replaced every 3 days until the cells reached confluence. Prior to trypsin/EDTA treatment (0.05%/0.02%, 2 min) cells were washed twice with PBS. Trypsin activity was neutralized by the addition of a 4-fold excess DMEM-F12. The resulting cell suspension was centrifuged at 300 *g* for 5 min. The cells pellet was resuspended in DMEM-F12 and plated in a 1:3–1:5 ratio. Studies were performed in cells after four to six passages.

Sphingolipid measurements

Sphingolipids from rat small bowel tissue were analyzed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) as described previously (17).

The internal standards for the mass spectrometric analyses were C17-sphingosine (d17:1), C17-sphingosine1-phosphate, and C17-sphinganine-1-phosphate, C12-ceramide (N-dodecanoyl-sphingosine, d18:1/12:0), C12-ceramide 1-phosphate, and C12-sphingomyelin. Internal standards were prepared as 1 mM stocks in ethanol and were mixed to produce a cocktail that contained each compound at 50 μM (to deliver 500 pmol in 10 μl). These stocks could be stored for at least several months in microfuge tubes (1.5 ml homo-polymer microtubes, MCT-150-C, Axygen Scientific, Union City, CA, USA) and were checked periodically for losses and replaced when needed.

Prior to extraction, tissues were homogenized in ice-cold PBS, then transferred into 13 × 100 mm borosilicate screw cap test tubes with Teflon™ caps. The aqueous volume at this stage was 0.1 to 0.2 ml. After adding 0.5 ml of methanol and the internal standards (in 10 μl), the lipids were dispersed using a bath-type sonicator (Bronson 1510 ultrasonicator) at room temperature for 30 s, 0.25 ml of chloroform was added and the capped tubes were incubated at 48°C overnight in a heating block. After cooling, 75 μl of 1 M KOH in CH_3OH was added, mixed briefly by sonication, incubated in a heating block for 2 h at 37°C, and centrifuged to clarify. For analysis of sphingoid bases and related compounds, 0.4 μl was removed and transferred to a new test tube; the solvent was removed under vacuum at room temperature (in a Savant Speedvac), then the lipids were redissolved in the LC mobile phase for reverse phase chromatography with sonication, centrifuged to clarify, and transferred to autoinjector vials. The remainder of the extract was neutralized with 3 μl of glacial acetic acid, and 1 ml of $CHCl_3$ and 2 ml of H_2O were added. The samples were mixed, then centrifuged, the lower layer was removed with care to recover the interface, transferred to a new test tube, and the solvent was removed under vacuum at room temperature (in a Savant Speedvac), then the lipids were redissolved in the LC mobile phase for normal phase chromatography with sonication, centrifuged to clarify and transferred to autoinjector vials.

Sphingoid bases and 1-phosphates (including ceramide 1-phosphate) were separated by reverse-phase HPLC using a binary system (Perkin Elmer Series 200 MicroPump) and a Supelco 2.1 mm i.d. × 5 cm Discovery C18 column and a flow rate of 1 μl/min. Mobile phase A consisted of CH_3OH: H_2O: HCOOH (58:41:1) (all mobile phase solvents are given in v:v); mobile phase B consisted of CH_3OH:HCOOH (99:1); both also contained 5 mM ammonium formate. For each analysis, the column was equilibrated with 60:40 (A:B) for 0.4 min, the sample was injected (50 μl by a Perkin Elmer Series 200 Autosampler) and 60:40 (A:B) was continued for 0.5 min., followed by a 1.8 min linear gradient to 100% B, which was held for 5.3 min, then the column was re-equilibrated at initial conditions for 0.5 min.

Complex sphingolipids (Cer, SM) were separated by normal phase chromatography using a Supelco 2.1 mm i.d. × 5 cm LC-NH_2 column and a flow rate of 1.0 μl/min. Mobile phase A consisted of CH_3CN: CH_3OH: CH_3COOH (97:2:1); mobile phase B consisted of CH_3OH:H_2O: $CH_3(CH_2)_3OH$: CH_3COOH (64:15:20:1); both also contained 5 mM ammonium acetate. For each analysis, the column was equilibrated with 98:2 (A: B) for 0.5 min, the sample was injected, and 98:2 (A: B) was continued for 1.1 min, followed by a 0.2 min. linear gradient to 82% A. This state is held for 0.4 min, followed by a 0.8 min linear gradient to 100% B and re-equilibration of the column at initial conditions for 0.5 min.

The mass spectrometry data was collected using a PE Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source. Dry N_2 was used as the nebulizing gas at a flow rate of 6 l/min. The ionspray needle was held at 5500 V, and the orifice and ring voltages were kept low (30–40 V and 180–220 V, respectively) to minimize collisional decomposition of molecular ions prior to entry into the first quadrupole, and the N_2 drying gas temperature was set to 500°C. N_2 was used to collisionally induce dissociations in Q2, which was offset from Q1 by 30–40 V. Q3 was then set to pass molecularly distinctive product ions. A multiple reaction monitoring method (MRM) has been developed for the typical fatty acyl chain length variants for these mammalian sphingolipids (i.e., C16:0, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1, and C26:0), including variants with a 4-hydroxysphinganine backbone (17). With the typical number of subspecies for each compound, each transition had a dwell time of ~25 ms. After building the MRM protocol, the intensities of the signals for the different sphingolipid subspecies were compared with the internal standards to arrive at estimations of the mass usually within ± 10%.

PGE_2 measurement

Secreted PGE_2 was assayed using the prostaglandin E2 monoclonal EIA kit from Cayman Chemical (Ann Arbor, MI, USA) according to manufacturer's instructions and measured at 412 nm.

Immunocytochemistry

Expression of α-actin, a smooth muscle contractile protein, was assessed by fluorescence microscopy. Cells were plated on glass coverslips for up to 72 h. When cells were 50–70% confluent, they were fixed with cold methanol for 10 min at –20°C. Fixed cells were washed three times with PBS and blocked by incubation in PBS with 3% BSA for 1 h at room temperature. Cells were washed once with 0.3% PBS/BSA, then incubated overnight at 4°C with a smooth muscle α-actin primary Ab (1:100). Coverslips were washed three times with PBS, then incubated for 1 h at room temperature with secondary Ab conjugated to green fluorescent Alexa Flour 488 dye. Immunoreactive protein was visualized by fluorescence microscopy.

Immunoblot analysis

Cells were harvested in ice-cold PBS, centrifuged (2000 *g*, 10 min, 4°C) and incubated with lysis buffer containing 20 mM HEPES (pH 7.4), 100 mM sodium chloride, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 50 mM β-glycerophosphate, 2 mM sodium orthovanadate, 2 mM EDTA, and Complete Mini Protease Inhibitor (1 tablet for 10 ml buffer), followed by sonication for 15 s. Cell debris were removed by centrifugation for 10 min at 10,000 *g* at 4°C. Total cellular protein (10 μg) was separated on 10% SDS-polyacrylamide gels (PAGE), then blotted onto enhanced nitrocellulose membranes. Membranes were blocked with a buffer containing nonfat dry milk (5% w/v) and Tween-20 (0,05% v/v) in case of COX-2 or with RotiBlock in case of MAPKs for 1 h at room temperature. Afterward the membranes were incubated with the respective primary Ab diluted in blocking solution for COX-2 or Tris-buffered NaCl solution (TBS, 137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 for MAPKs overnight at 4°C. Antibodies specific for phosphorylated or total ERK and p38 MAPK were diluted 1:10,000 and the Ab against COX-2 was diluted 1:400. The membranes were rinsed with TBS containing 0.1% Tween-20, and incubated for 2 h at room temperature in either horseradish peroxidase (HRP) -conjugated goat anti-rabbit IgG Ab (diluted 1:10,000 in TBS with 0.1% Tween-20) in case of MAPKs or in a HRP-conjugated protein G (diluted as above) in case of COX-2. Bound antibodies were visualized using enhanced chemiluminescence (ECL) on X-ray films.

To confirm equal amounts of protein loaded for each sample, blots were subsequently stripped using a low pH buffer containing 0.2 M glycine, pH 2.2, 0.1% SDS, and 1% Tween-20 and reprobed with an Ab specifically recognizing total (phosphorylated and unphosphorylated) MAPKs or α-tubulin for membranes first treated with COX-2 Ab.

Western blots were densitometrically evaluated using the facilities of the AlphaDigiDoc gel documentation system (Bio-Rad, München, Germany).

Quantification of arachidonic acid (AA) release

Cells were labeled overnight with 0.2μCi/ml of [^{14}C] AA in culture medium. Then medium was removed, cells were rinsed three times with culture medium, then incubated in 1 ml of medium containing the desired additives. After incubation for the indicated times, medium was removed and an aliquot was submitted to scintillation counting. Results were normalized to cell protein. In addition, media were extracted with organic solvents and subjected to thin-layer chromatography (TLC). In brief, 500 μl of medium were transferred to a glass tube and 3.75 ml of $CHCl_3/CH_3OH$ (1:2, by volume), 1,25 ml $CHCl_3$, and 1.25 ml 0.2% fumaric acid were added successively. After vortexing the tubes were centrifuged for 5 min, 1800 *g*, 4°C for phase separation. The lower organic phase was applied on Silicagel-60 TLC plates developed with n-hexane/diethyl ether/glacial acetic acid (70:30:1, by volume). AA was visualized by autoradiography using the bioimaging analyzer Fujix bas 1000 software, Tina 2.09, (Raytest, Straubenhardt, Germany).

Protein determination

Cell protein was quantified as described by Bradford (18) using BSA as a standard.

RESULTS

The contents of S1P and of C1P are increased in the *tunica muscularis externa* after intestinal manipulation of rats

The content of several SLs, including sphingomyelin (SM), ceramide, sphingosine, C1P, and S1P was analyzed in intestinal tissue from rats subjected to a standardized, nontraumatic intestinal manipulation. At different time intervals following surgery, muscularis and mucosa layers were collected and subjected to lipid analysis using ESI tandem mass spectrometry. At time zero the amount (mol/mg wet wt) of all lipids analyzed, including all detectable molecular subspecies, was higher in the muscle layer than in mucosa. SM was most abundant with ~500 and 250 pmol/mg, followed by ceramide with almost 300 and 100 pmol/mg in muscularis and mucosa, respectively. In both layers the amount of sphingosine was by one order of magnitude below the mass of ceramide, while SL-phosphates, C1P and S1P ranged in a picomolar region. As illustrated in **Fig. 1** the most abundant subspecies of all N-acylated SLs analyzed, including SM, ceramide, and C1P, were C16 and C18, followed by C24:1 and C20 (Fig. 1). Whereas no significant changes of any of the SLs analyzed have been observed in the mucosa layer after intestinal manipulation (data not shown), the muscularis exhibited a time-dependent increase in the content of C1P and of S1P (**Fig. 2**). As illustrated in Fig. 2 the time courses of expression of the two SL phosphates were quite similar. Both peaked at 6 h after intestinal manipulation, reaching a >3-fold higher level at this time point than controls. After 6 h the amount of C1P decreased slowly but continuously toward control values, whereas that of S1P first decreased, then remained increased to some extent at longer intervals. Notably, the amount of both SLs appears to be subjected to a long-time up-regulation postsurgery.

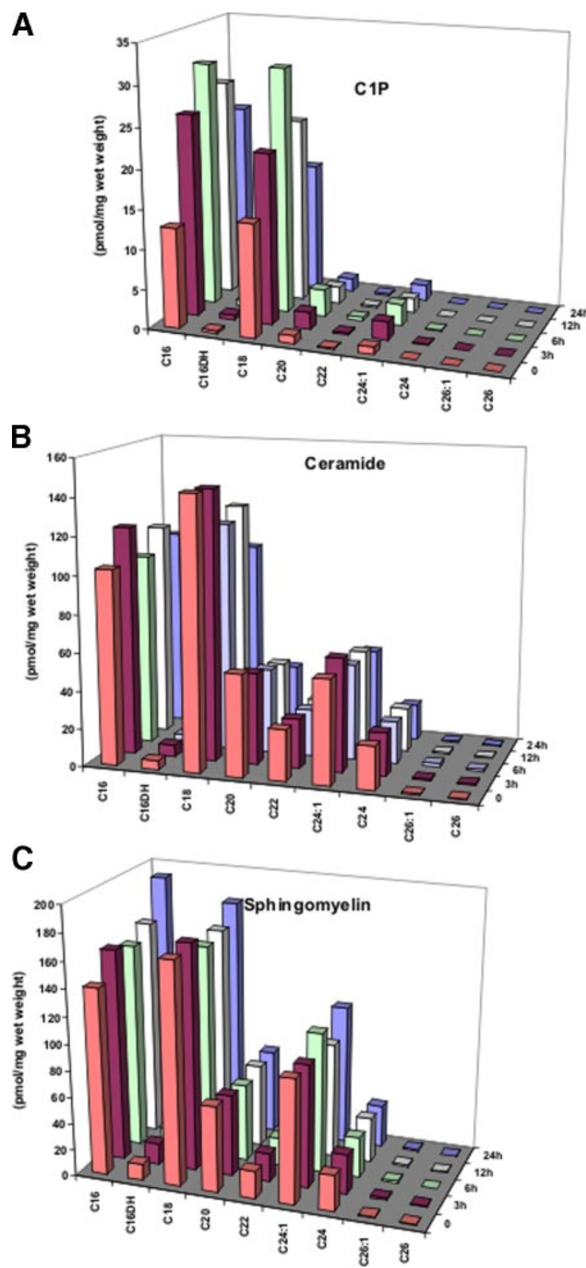
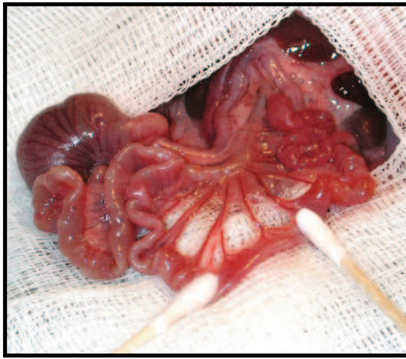


Figure 1. The content of sphingolipids in the intestinal muscularis after mechanical stress. Rat small intestine was gently manipulated and the muscularis layer was collected at the indicated time points after surgery. Sphingolipids were extracted and the indicated species including all detectable subspecies, respectively, were determined by ESI tandem mass spectrometry as described in Materials and Methods. Numbers indicate carbon chain length followed by the number of double bonds in the fatty acid. Data are averages of triplicate determinations and expressed as pmol lipid/mg wet wt.

Characterization of cultured rat smooth muscle cells

Smooth muscle cells represent the major cell type in the intestinal muscularis layer and their contractility is



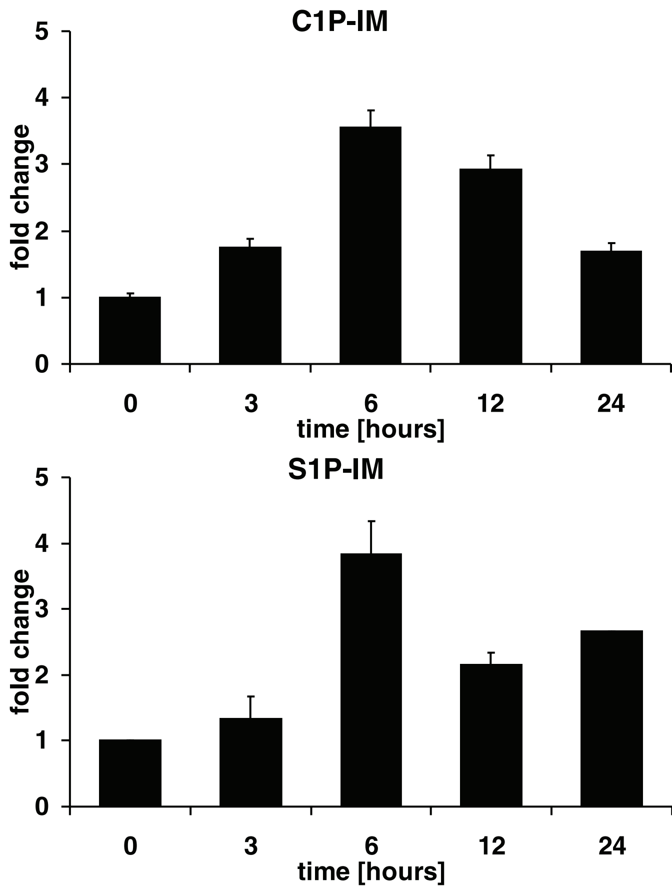


Figure 2. Time-dependent increase of C1P and S1P amounts in the muscularis layer of small bowel after intestinal manipulation (IM). Rat small intestine was gently manipulated with moist cotton applicators and the muscularis layer was collected at the indicated time points after surgery. Sphingolipids were extracted and C1P and S1P analyzed by mass spectrometry as described in Materials and Methods. Data are averages of triplicate measurements and expressed relative to control values obtained at time 0. Note that the amount of S1P determined at 24 h was not significantly different from that at 12 h as assessed by Student's t test ($P<0.05$).

primarily affected after abdominal surgery (11). We have therefore established a primary culture of rat intestinal smooth muscle cells and examined the involvement of exogenous S1P and C1P in the potential proinflammatory response of these cells. Smooth muscle cells from the circular and the longitudinal muscle layer of rat small bowel were isolated by enzymatic digestion and cultured through five or six passages. A homogeneous culture of smooth muscle cells was achieved by density gradient centrifugation, thus separation from other cell types that reside in the intestinal muscle layer, such as enteric neurons, macrophages and dendritic cells. To verify the smooth muscle origin and homogeneity of the cultures, we tested the expression of α-actin, a typical smooth muscle contractile protein. As shown in **Fig. 3** semiconfluent grown cells clearly exhibited immunoreactivity for smooth muscle α-actin, thus supporting the smooth muscle origin of our cell cultures.

S1P and C1P stimulate PGE_2 formation in primary cultured intestinal smooth muscle cells

Considering that PGE_2 has emerged as the prototypical eicosanoid with key functions in acute inflammatory responses (19, 20), we investigated the effect of S1P and of C1P on PGE_2 formation in primary cultured intestinal smooth muscle cells. As shown in **Fig. 4*A***, both SLs clearly enhanced PGE_2 formation. S1P induces a pronounced but short increase in PGE_2 formation at 4 h (Fig. 4*C*), whereas in the presence of C1P, synthesis of

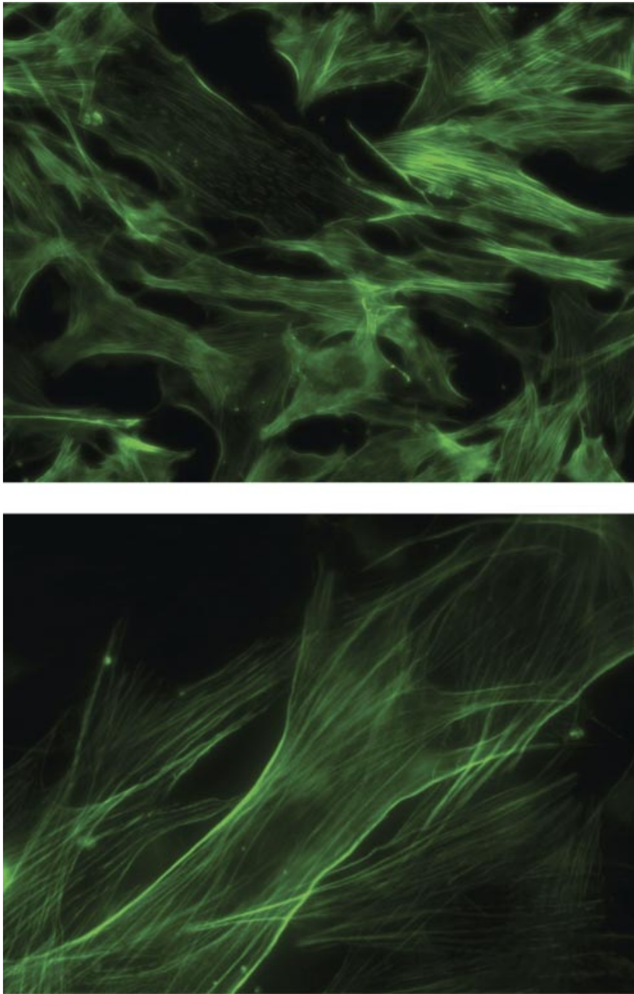


Figure 3. Expression of smooth muscle contractile protein, α-actin in primary cultured rat intestinal smooth muscle cells. Cells plated on glass coverslips were grown to 50% confluence, fixed, and stained with Ab against smooth muscle α-actin isoform (1:100). Antimouse secondary Ab conjugated to green fluorescent Alexa Flour 488 dye was used at 1:200. Immunoreactivity was visualized by fluorescence microscopy (×40; *A*), (×63; *B*).

PGE_2 ascends continuously for up to 24 h (Fig. 4*D*), returning to control levels at later time points (30 and 48 h, not shown).

S1P stimulates COX-2 induction whereas C1P stimulates arachidonic acid (AA) release in primary cultured intestinal smooth muscle cells

Previous studies have shown that COX-2 induction is responsible for an increased synthesis of PGE_2 in response to S1P in different cell lines (7, 21). Moreover, it has been reported that PGE_2 production via COX-2 is a causative mechanism of postoperative ileus (22). We therefore examined the expression of COX-2 protein in the presence of C1P and of S1P in primary cultured intestinal smooth muscle cells. As becomes clear from Fig. 4*A*, C1P did not affect COX-2 expression whereas a considerable increase in the 72 kDa COX-2 protein had already been observed in the presence of S1P at a concentration of 1 μM (Fig. 4*B*). This effect was only somewhat higher in the presence of S1P at 5- and 10-fold higher concentrations. The time course of S1P-induced COX-2 expression revealed quite a fast effect, which increased continuously with time reaching a maximum on average of 400% ± 60% by 4 h (Fig. 4*C*). Note the similarity of the curves illustrating the time dependency of the effect of S1P on COX-2 expression and on PGE_2 formation, respectively.

Since C1P clearly stimulated PGE_2 production but had no effect on COX-2 expression, we investigated its effect on AA release. Liberation of AA from membrane phospholipids is catalyzed by PLA_2, and represents the initial and rate-limiting step in PGE_2 synthesis (2).

For this purpose, we labeled intestinal smooth muscle cells with $[^{14}C]$-AA, then analyzed its release in the absence (control cells) and presence of C1P. As shown in Fig. 4*D*, the amount of AA released from the cells increased in a time-dependent manner up to 3-fold at 24 h in the presence of C1P compared with control cells and returning to control levels at later time points (30 and 48 h, not shown). As determined by TLC, the data shown in Fig. 4*D* refer only to AA itself and not to AA-labeled lipids. Related lipids, including S1P, phosphatidic acid, and ceramide, did not affect AA release in primary cultured intestinal smooth muscle cells (data not shown), suggesting the specificity of C1P action.

S1P-induced COX-2 expression is pertussis toxin (PTX) sensitive and mediated by MAPKs in primary cultured intestinal smooth muscle cells

It is well known that S1P can act either as an extracellular ligand by binding to a family of G-protein-coupled receptors or as an intracellular second messenger with so far unknown direct targets (23). All five members of the S1P receptor family can be coupled to $G_{i/0}$ proteins (24). Since the latter are inactivated by PTX via ADP-ribosylation, we used this toxin to determine in a first approach whether the stimulatory effect of S1P on COX-2 expression is receptor-mediated. As shown in **Fig. 5*A***, PTX clearly reduces S1P-induced COX-2 expression. However, there is considerable stimulation of COX-2 by PTX alone. This result once more suggests the complexity of COX-2 expression. Multiple signaling pathways have been implicated in the regulation of COX-2 expression in different cell types (21, 25). In the present study we examined whether MAPKs are involved in S1P induced COX-2 expression. As shown in Fig. 5*B*, S1P induced a transient activation of ERK. Moreover, COX-2 expression was clearly reduced in the presence of PD98059, a specific inhibitor of this MAPK pathway (26). Similar results were obtained with SB239063, known to specifically inhibit p38 MAPK (Fig. 5*C*). By contrast, stress-activated protein kinase (SAPK) was insensitive to S1P administration and no change of S1P-induced COX-2 expression has been observed in

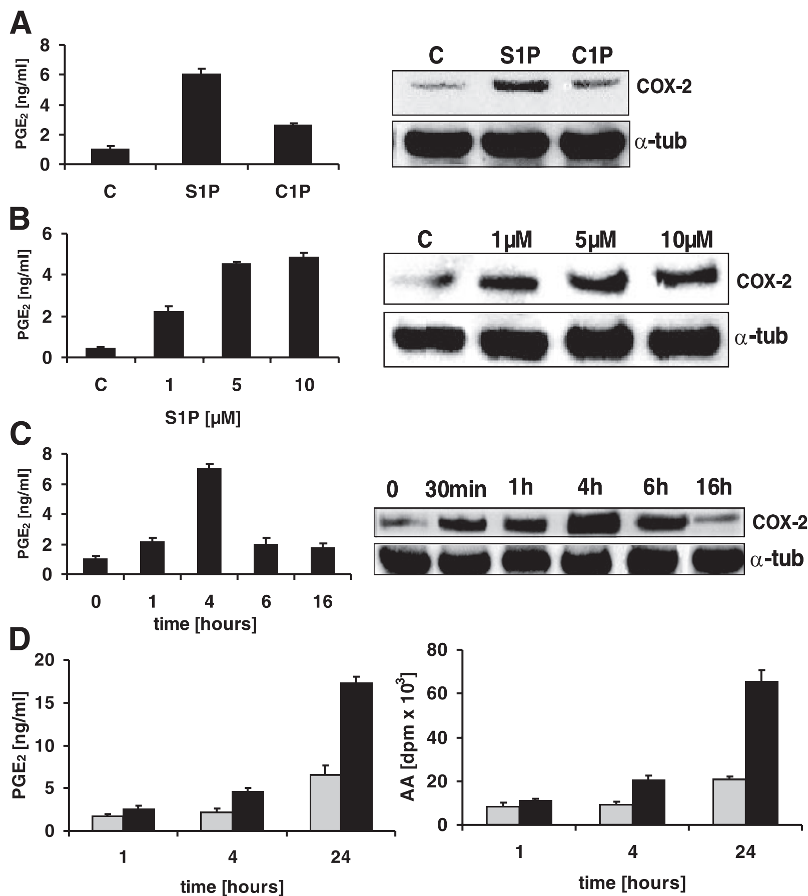


Figure 4. Effects of S1P and of C1P on PGE_2 synthesis on COX-2 induction and on AA release in primary cultured rat intestinal smooth muscle cells. *A)* Cells were incubated for 4 h in the presence of 10 μM of S1P or C1P or *B)* with the indicated concentrations of S1P for 4 h. *C)* Alternatively, cells were incubated with 10 μM of S1P for the indicated times. Control cells (C) were always treated accordingly in the presence of vehicle. PGE_2 was measured in the media as described in Materials and Methods. Data are means ± SD from 3 independent experiments. COX-2 was determined in lysed cell protein (10 μg) and subjected to 10% SDS-PAGE and immunoblot analysis, as described in Materials and Methods. An anti- ∝-tubulin (∝-tub) Ab was used to confirm equal loading. The blots shown are from one representative of at least 3 independent experiments. *D)* Cells were labeled overnight with 0.2 μCi [^{14}C]-AA (arachidonic acid)/ml culture medium. Then medium was removed and cells rinsed three times with culture medium prior to incubation in the presence of vehicle (controls, gray columns) or of 10 μM of C_8-C1P (black columns) for the indicated times. Results were normalized to cell protein levels. Data are means ± SD from 3 independent experiments.

the presence of the SAPK inhibitor SP600125 (results not shown).

DISCUSSION

In the present study we found that an increase in S1P and C1P occurs only in the intestinal muscle layer, and not in the mucosa from rats subjected to a standardized surgical manipulation. This finding is remarkable, and indicates for the first time a direct involvement of these two bioactive lipids in the local inflammatory response within the intestinal muscularis, which has been suggested to play a major role in the pathogenesis of postoperative ileus (11, 15). Even more remarkable is the finding that primary cultured intestinal smooth muscle cells exhibit a proinflammatory activity in the presence of C1P and S1P. Regardless of the complexity of the inflammatory response within intestinal muscularis, probably involving several cell types, our study demonstrates that S1P and C1P stimulate PGE_2 formation in intestinal smooth muscle cells (**Fig. 6**). The latter represent not only the prevailing cell type of the intestinal muscle layer, but have been shown to be mostly affected in their function, as suggested by the well-documented postoperative dysmotility (11, 15). The role of S1P and C1P in inflammation appears to be closely related to the metabolism of eicosanoids (2). It was therefore not surprising that S1P and C1P modulate PGE_2 synthesis. But it was of some surprise that modulation of PGE_2 synthesis by these bioactive lipids, previously described primarily in immune cells and diverse cell lines (2, 27), is also valid in primary cultured intestinal smooth muscle cells. It was suggested recently that C1P and S1P may act in concert to regulate PGE_2 production (10, 27). Our findings strongly support this suggestion because of *1)* the similarity of the time course concerning the increase of the level of both SL metabolites in the muscle layer after intestinal manipulation, *2)* stimulation of COX-2 expression and AA release by S1P and C1P, respectively, and *3)* the early but transient vs. a continuously ascending increase of PGE_2 formation induced by S1P and C1P, respectively. The latter finding also supports the hypothesis that S1P primes the system for PGE_2 synthesis by inducing COX-2 (27). We cannot, however, exclude the possibility that known metabolic instability of S1P is explicative for the transience of all the effects observed in the presence of this bioactive agent.

Smooth muscle cells have been shown to exhibit an inflammatory response to exogenous S1P (28). In those cells isolated from human trachea, modulation of cytokines by S1P was PTX independent, indicating it did not involve G-protein-coupled receptors. By contrast, in rat intestinal smooth muscle cells the PTX-sensitivity of S1P-induced COX-2 expression implicates the involvement of $G_{i/0}$-coupled S1P receptor(s). Based on several previous studies (for a review, see ref 24), we assume

that $S1P_2$, which has been found to play a key role in myogenic differentiation (29), also mediates S1P-induced COX-2 expression in intestinal smooth muscle cells. Note that we have identified $S1P_2$ by RT-polymerase chain reaction (RT-PCR) in rat intestinal smooth muscle cells (result not shown). S1P receptor-mediated effects often imply MAPK signaling pathways. The ERK MAPK pathway usually promotes cell proliferation whereas activation of SAPK/c-Jun NH_2-terminal kinase (JNK) is important in controlling apoptosis, and p38 MAPKs appear to be key regulators of inflammatory response in immune cells (30). Due to its tumor-promoting function, inflammation is often considered a proliferative rather than an apoptotic process (27). Thus, one would imply activation of the ERK signaling pathway upstream of COX-2 expression. This actually proved to be true for S1P-modulated COX-2 expression in human amnion-derived WISH cells (21) and also in human coronary artery smooth muscle cells (25). However, S1P-induced COX-2 expression in intestinal smooth muscle cells appears to involve phosphorylation of both ERK and p38 MAPK signaling pathways. Remarkably, a similar result was obtained for the promyogenic and antiproliferative $S1P_2$-mediated action of S1P in myoblasts (29). It thus appears that S1P induces two opposite physiological processes, namely morphogenesis and inflammation, albeit in undifferentiated myoblasts and differentiated smooth muscle cells, respectively, using similar signaling pathways. This finding once more emphasizes that similar signaling pathways can induce different cellular responses depending on the developmental stage of the cell.

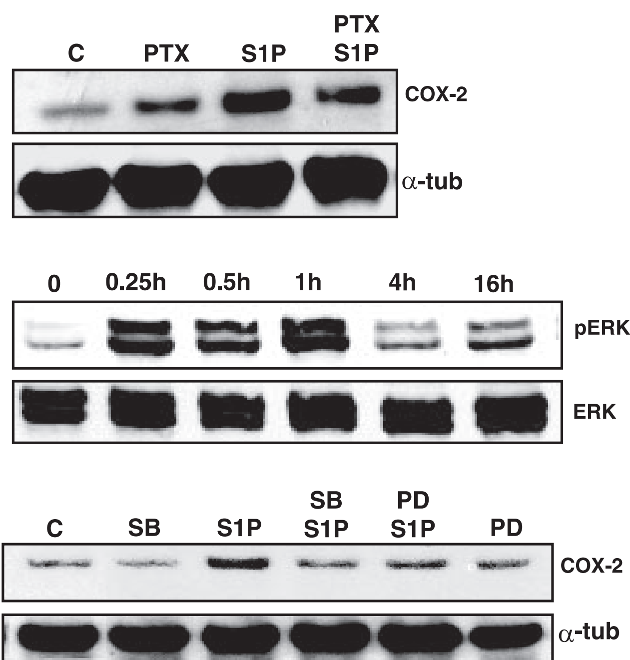


Figure 5. Effects of PTX and of MAPK inhibitors on COX-2 expression in primary cultured rat intestinal smooth muscle cells. *A)* Cells were preincubated for 2 h in medium containing 100 ng/ml PTX, prior to being treated with 10 μM S1P. Controls (C) were treated accordingly using vehicle. *B)* Cells were incubated with 10 μM S1P for the indicated times and phosphorylated ERK was determined by immunoblot analysis using antiphospho-ERK Ab. *C)* Cells were treated with vehicle (C), 30 μM of PD98059 (PD), or 5 μM of SB239063 (SB) for 30 min prior to incubation with 10 μM S1P. After 4 h, cell protein was lysed and subjected to SDS-PAGE and immunoblot analysis, as described in Materials and Methods. The blots shown are from one representative of 3 independent experiments.

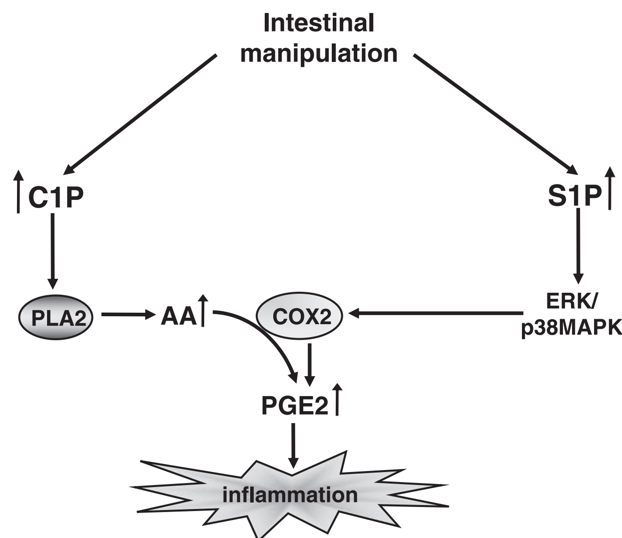


Figure 6. Scheme of proposed mechanism of sphingolipid involvement in inflammatory responses generated by surgical manipulation of the bowel.

In this study we identified C1P and S1P as potential inflammatory mediators following minor intestinal manipulation. We also showed that intestinal smooth muscle cells are targets of these bioactive sphingolipids. The fact that C1P and S1P stimulate synthesis of PGE_2, a prototypical eicosanoid with key functions in acute and chronic inflammation in these cells, makes them interesting targets for the development of novel anti-inflammatory drugs.

We thank Prof. K. Sandhoff for continual support as departmental chair, Mariola Lysson for excellent technical assistance, and Roland Broere for help in preparing the figures. This study was supported by the Deutsche Forschungsgemeinschaft (KFO 115) and for the mass spectrometric analyses by NIH grant GM069338 for the Sphingolipid Core of the Lipid MAPS Consortium (A.H.M.).

REFERENCES

1. Luckey, A., Livingston, E., and Taché, Y. (2003) Mechanisms and treatment of postoperative ileus. *Arch. Surg.* **138,** 206–214
2. Pettus, B. J., Chalfant, C. E., and Hannun, Y. A. (2004) Sphingolipids in Inflammation: roles and Implications. *Curr. Mol. Med.* **4,** 405–418
3. Turini, M. N., and DuBois, R. N. (2002) Cyclooxygenase-2: a therapeutic target. *Annu. Rev. Med.* **53,** 35–57
4. Spiegel, S., and Merrill, A. H., Jr. (1996) Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10,** 1388–1397
5. Ballou, L. R., Barker, S. C., Postlethwaite, A. E., and Kang, A. H. (1990) Sphingosine potentiates IL-1-mediated prostaglandin E2 production in human fibroblasts. *J. Immunol.* **145,** 4245–4251

6. Ballou, L. R., Chao, C. P., Holness, M. A., Barker, S. C., and Raghow, R. (1992) Interleukin-1-mediated PGE2 production and sphingomyelin metabolism. Evidence for the regulation of cyclooxygenase gene expression by sphingosine and ceramide. *J. Biol. Chem.* **267,** 44–50
7. Pettus, B. J., Bielawski, J., Porcelli, A. M., Reames, D. L., Johnson, K. R., Morrow, J., Chalfant, C. E., Obeid, L. M., and Hannun, Y. A. (2003) The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha. *FASEB J.* **17,** 1411–1421
8. Pettus, B. J., Bielawska, A., Spiegel, S., Roddy, P., Hannun, Y. A., and Chalfant, C. E. (2003) Ceramide kinase mediates cytokine- and calcium ionophore-induced arachidonic acid release. *J. Biol. Chem.* **278,** 206–213
9. Pettus, B. J., Bielawska, A., Subramanian, P., Wijesinghe, D. S., Maceyka, M., Leslie, C. C., Evans, J. H., Freiberg, J., Roddy, P., Hannun, Y. A., and Chalfant, C. E. (2004) Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. *J. Biol. Chem.* **279,** 320–326
10. Pettus, B. J., Kitatani, K., Chalfant, C. E., Taha, T. A., Kawamori, T., Bielawski, J., Obeid, L. M., and Hannun, Y. A. (2005) The coordination of prostaglandin E2 production by sphingosine-1-phosphate and ceramide-1-phosphate. *Mol. Pharmacol.* **68,** 330–335
11. Kalff, J.C., Buchholz, B. M., Eskandari, M. K., Hierholzer, C., Schraut, W.H., Simmons, R. L., and Bauer, A. J. (1999) Biphasic response to gut manipulation and temporal correlation of cellular infiltrates and muscle dysfunction in rat. *Surgery* **126,** 498–509
12. Wehner, S., Schwarz, T. N., Hundsdoerfer, R., Hierholzer, C., Tweardy, D. J., Billiar, T. R., Bauer, A. J., and Kalff, J. C. (2004) Induction of IL-6 within the rodent intestinal muscularis after intestinal surgical stress. *Surgery* **137,** 436–446
13. Khan, I., Blennerhassett, M. G., Kataeva, G. V., and Collins, S. M. (1995) Interleukin1 beta induces the expression of interleukin 6 in rat intestinal smooth muscle cells. *Gastroenterology* **108,** 1720–1728
14. Salinthone, S., Singer, C. A., and Gerthoffer, W. T. (2004) Inflammatory gene expression by human colonic smooth muscle cells. *Am. J. Physiol.* **287,** G627–G637
15. Kalff, J. C., Schraut, W. H., Simmons, R. L., and Bauer, A. J. (1998) Surgical manipulation of the gut elicits an intestinal muscularis inflammatory response resulting in postsurgical ileus. *Ann. Surg.* **228,** 652–663
16. Eskandari, M. K., Kalff, J. C., Billiar, T. R., Lee, K. K., and Bauer, A. J. (1997) Lipopolysaccharide activates the muscularis macrophages network and suppresses circular smooth muscle activity. *Am. J. Physiol.* **273,** G727–734
17. Merrill, A. H., Jr., Sullards, M. C., Allegood, J. C., Kelly, S., and Wang, E. (2005) Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods* **36,** 207–224
18. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254
19. Dempke, W., Rie, C., Grothey, A., and Schmoll, H. J. (2001) Cyclooxygenase-2: a novel target for cancer chemotherapy? *Cancer Res. Clin. Oncol.* **127,** 411–417
20. Kuehl, F. A., and Egan, R. W. (1980) Prostaglandins, arachidonic acid, and inflammation. *Science* **210,** 978–984
21. Kim, J. I., Jo, E.J., Lee, H-Y., Cha, M. S., Min, K. J., Choi, C. H., Lee, Y. M., Choi, Y-A., Baek, S-H., Ryu, S. H., Lee, K. S., Kwak, J-Y., and Bae, Y-S. (2003) Sphingosine 1-phosphate in amniotic fluid modulates cyclooxygenase-2 expression in human amnion-derived WISH cells *J. Biol. Chem.* **278,** 731–736
22. Schwarz, N. T., Kalff, J. C., Türler, A., Engel, B. M., Watkins, S. C., Billiar, T. R., and Bauer, A. J. (2001) Prostanoid production via COX-2 as a causative mechanism of rodent postoperative ileus. *Gastroenterology* **121,** 1354–1371
23. Spiegel, S., and Milstien, S. (2003) Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. *Biochem. Soc. Trans.* **31,** 1216–1219
24. Ishii, I., Fukushima, N., Ye, X., and Chun, J. (2004) Lysophospholipid receptors: signaling and biology. *J. Biol. Chem.* **73,** 321–354
25. Damirin, A., Tomura, H., Komachi, M., Tobo, M., Sato, K., Mogi, C., Nochi, H., Tamoto, K., and Okajima, F. (2005) Sphingosine 1-phosphate receptors mediate the lipid-induced cAMP accumulation through cyclooxygenase-2/prostaglandin i_2 pathway in human coronary artery smooth muscle cells. *Mol. Pharm.* **67,** 1177–1185
26. Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J. Biol. Chem.* **270,** 489–494
27. Chalfant, C. E., and Spiegel, S. (2005) Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. *J. Cell Sci.* **118,** 4605–4612
28. Ammit, A. J., Hastie, A. T., Edsall, L. C., Hoffman, R. K., Amrani, Y., Krymskaya, V. P., Kane, S. A., Peters, S. P., Penn, R. B., Spiegel, S., and Panettieri, R. A., Jr. (2001) Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J.* **15,** 1212–1214
29. Donati, C., Meacci, E., Nuti, F., Becciolini, L., Farnararo, M., and Bruni, P. (2004) Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor. *FASEB J.* **19,** 449–451
30. Johnson, G. L., and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298,** 1911–1912

Received for publication January 30, 2006.
Accepted for publication April 26, 2006.