

**Proinflammatory action of sphingolipids in primary cultured rat
intestinal smooth muscle cells: implications for postoperative- and
sepsis-induced ileus**

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn 2007

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn.

The work for this PhD thesis was performed from March 2003 to December 2006 in the laboratory of PD. Dr. Gerhild van Echten-Deckert at the Kekulé Institute of Organic Chemistry and Biochemistry, Bonn, Germany.

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Tag der Prüfung: 26.03.2007

Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert.

Erscheinungsjahr: 2007

To my parents

my sister

Cristian

Acknowledgements

The following is my heartfelt appreciation to those people, both past and present, who gave me spirit and encouragement to start, conduct and complete this thesis:

First of all I would like to thank PD. Dr. Gerhild van Echten-Deckert for introducing me to the sphingolipid world and for giving me the chance to study my PhD under her highly skilled supervision. I am particularly grateful that she has never stopped believing in my ability and that she did not let me to leave. Thank you for the independence you gave me. I wish to thank you not only for the scientific support, for constructive criticism and careful revision of my manuscripts and of my PhD thesis but also for patience, trust and friendship. I truly appreciate your ability of doing a great career and at the same time having a beautiful family. This gives me confidence that some day I can also achieve this.

I want to extend my thanks to Prof. Dr. Konrad Sandhoff for giving me the opportunity to conduct the research at this institute.

My sincere thanks go also to Prof. Dr. Volker Herzog, for kindly accepting to be co-referee, for support and for evaluating the thesis.

I would like to thank Prof. Dr. Milan Höfer for his agreement to participate in my PhD committee.

I am grateful and indebted to Prof. Dr. Andreas Hirner and to Prof. Dr. Jörg Kalff for giving us the opportunity to work in such an interesting project, “Klinische Forschergruppe 115 der Deutschen Forschungsgemeinschaft”, for friendship and organizing such constructive and exciting scientific meetings.

I also want to thank Prof. Dr. Jörg Kalff for agreement to participate in my PhD committee and to his co-workers especially to Dr. Sven Wehner, Bastian Lüdenbach, Mariola Lysson, Anatol Rocke for preparing tissue for our studies, for help with the isolation of smooth muscle cells and with stretching experiments.

My thanks go to my present and former colleagues in the research team- Annika Wedeking, Roland Broere, Nadine Hagen, Sven Nätzker, Lars Niederhausen, Cristian Gurgui, Simone Brachtendorf, Gemma Triola, Dana Becker for a nice working environment, interesting discussions and lots of fun. Annika, vielen Dank for giving me a helping hand whenever I had computer issues. Sven, thanks for teaching me immunoblotting. Roland, danke for the help with molecular biology techniques, you are a good teacher.

Ein grosses Danke schön for our excellent technical assistant, Andrea Rath for introducing me to the cell culture techniques, for friendship and for stimulating me to practice my German.

A big thank to Ana Kralj, our labmate from time to time, not only for her support and encouragement during these years but especially for her friendship, her wonderful and delicious cakes and dinners. We spent nice time together in the lab and outside. I will never forget our scientific and private discussions. I will miss you.

I would like to thank to all AK Sandhoff group members that I worked with during these years for help and nice atmosphere.

I could have not reached this stage of my life without meeting great teachers throughout my studies. I am particularly thankful to my chemistry teacher from high school, Mrs. Tania Teodorescu and to my Diploma supervisor, Prof. Dr. Anca Dinischiotu for giving me the chance to complete my Master studies at University of Bonn as Erasmus student.

Thanks to all my friends, especially to those I did not mention here.

Special thanks go to my husband Cristian, who always believed in me and supported me in every possible way. Thank you for proofreading the thesis and for useful comments and suggestions. I would not have reached here without your support as well. Finally, I want to extend my heartfelt thanks to my parents and my sister who always believed in me and gave me the courage and confidence to follow my way. Thanks for your endless love, care, support and coping so great with the distance that is between us.

Scientific Work

Publications

van Echten-Deckert G., Dragusin M. (2007) Golgi localization of glycosyltransferases involved in ganglioside biosynthesis. *Curr Drug Targets: (invited review submitted)*

Dragusin M., Wehner S., Kelly S., Wang E., Merrill AH Jr., Kalff J.C., van Echten-Deckert G. (2006) Effects of sphingosine-1-phosphate and ceramide-1-phosphate on rat intestinal smooth muscle cells: implications for postoperative ileus. *FASEB J: 20(11): 1351-9.*

Triola G., Fabrias G., Dragusin M., Niederhausen L., Broere R., Llebaria A., and van Echten-Deckert G. (2004) Specificity of the dihydroceramide desaturase inhibitor N-[(1R,2S)-2-hydroxy-1-hydroxymethyl-2-(2-tridecyl-1-cyclopropenyl)ethyl]octanamide (GT11) in primary cultured cerebellar neurons. *Mol Pharmacol: 66(6): 1671-8.*

Dragusin M., Gurgui C., Schwarzmann G., Hoernschemeyer J., and van Echten-Deckert G. (2003) Metabolism of the unnatural anticancer lipid safinol (L-threo-dihydrosphingosine) in cultured cells. *J Lipid Res: 44(9): 1772-9.*

Talks

”Effects of sphingosine-1-phosphate and ceramide-1-phosphate on rat intestinal smooth muscle cells: implications for postoperative ileus”, at **Symposium: From Proteomics to Lipidomics - Basics, Advances and Applications, Bonn, Germany (July, 2006)**

“Involvement of bioactive sphingolipids in the postoperative intestinal trauma”, at the **4th International Meeting of the Sphingolipid Club, University Residential Centre of Bertinoro, Forli, Italy (June, 2005) (Young investigator award)**

Poster presentations

“*Involvement of sphingolipids in inflammatory processes during postoperative intestinal trauma*”, at the 41st Congress of the European Society for Surgical Research, Rostock (May 2006) Authors: Dragusin M., Broere R., Wehner S., Kalff J.C., van Echten-Deckert.

“*Involvement of bioactive sphingolipids in the postoperative intestinal trauma*”, at the 3rd International Charleston Ceramide Conference, Charleston, Wild Dunes Resort Isle of Palms, USA (March 2005). Authors: Dragusin M., Wehner S., Kalff J.C., Speidel N., Schwarz N., van Echten-Deckert G.

“*Involvement of bioactive sphingolipids in the postoperative intestinal trauma*”, at the International Visceral Surgery Meeting focused on Intestine, Bonn, Germany (October 2004). Authors: Dragusin M., Wehner S., Kalff J.C., Speidel N., Schwarz N., Kelly S., Wang E., Alfred H. Merrill Jr., van Echten-Deckert G.

“*Metabolism of the unnatural anticancer lipid safinol (L-threo-dihydrosphingosine) in cultured cells*”, at the 2nd International Charleston Ceramide Conference, Como, Italy (June 2003). Authors: Dragusin M., Gurgui C., Schwarzmann G., Hoernschemeyer J., and van Echten-Deckert G. (*Poster Prize*)

Abbreviations

AP-1	Activator Protein
BSA	Bovine Serum Albumin
C1P	Ceramide-1-phosphate
CERT	Ceramide Transport Protein
CK	Ceramide kinase
COX	Cyclooxygenase
DEAE	Diethylaminoethanol
DMEM	Dulbecco's Modified Eagle Medium
DMS	Dimethylsphingosine
dpm	disintegrations per minute
ER	Endoplasmatic Reticulum
ERK	Extracellular-regulated kinase
g	grams
Gal	Galactose
GalCer	Galactosylceramide
Glc	Glucose
GlcCer	Glucosylceramide
GPCRs	G-protein-coupled receptors
h	hours
HBSS	Hank's balanced salt solution
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
Hz	Hertz
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukine
JNK	c-Jun NH ₂ -terminal kinases
LPS	Lipopolysaccharide
M	Molarity
mA	milliamper
MAPK	Mitogen-activated protein kinase
MC	Mucosa
ME	Muscularis
min	minutes
ml	millilitre
MS	Mass spectrometry
ms	millisecond
NF-kB	Nuclear factor-kappa B
nm	nanometer
NO	Nitric oxide
NSAID	Non-steroid anti-inflammatory drugs
PBS	Phosphate Buffered Saline
PD	PD98059, Inhibitor of ERK
PDGF	Platelet derived growth factor
PG	Prostaglandin
PGHS	Prostaglandin H synthases
PGT	Prostaglandin transporter
PLA ₂	Phospholipase 2

PTX	Pertussis-toxin
RISM	Rat intestinal smooth muscle
RP18	Reversed-phase 18
rpm	rotation per minute
RPMI	Roswell Park Memorial Institute, culture medium
RT	Room temperature
S1P	Sphingosine-1-phosphate
SAPK	Stress-activated protein kinase
SB	SB239063, Inhibitor of p38 MAPK
SDS	Sodium dodecylsulfate
SK	Sphingosine kinase
SM	Sphingomyelin
SMase D	Sphingomyelinase D
sn	second
TGF β	Transforming growth factor β
TLC	Thin layer chromatography
TLR	Toll like receptor
TNF- α	Tumor necrosis factor alpha
TNFRs	Tumor necrosis factor receptors
TXA ₂	Tromboxane 2
U	Units
UDP	Uridine diphosphate
UV	Ultraviolet
μ g	microgram
μ l	microlitre
V	Volt
VCAM	Vascular cell adhesion molecule

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Summary

Postoperative ileus or “paralysis of the bowel” is a transient but life-threatening impairment of bowel motility that occurs after surgery. Several pathogenic mechanisms responsible for postoperative dysmotility have been proposed including the involvement of autonomic nervous system, inhibitory humoral agents, anesthetic agents and local factors such as inflammation. Studies on inflammation progressively revealed a key role of sphingolipids in inflammatory responses. We have, therefore, investigated the involvement of sphingolipids in postoperative inflammation using a standardized surgical rodent model of intestinal manipulation.

Our data demonstrate for the first time a direct correlation between the bioactive sphingolipids, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), and postoperative intestinal inflammation. Sphingolipid analysis by mass spectrometry of both intestinal *muscularis* and *mucosa* after manipulation revealed a time dependent increase of S1P and C1P only in the *muscularis* layer when compared to control animals. We therefore examined the potential role of these two bioactive sphingolipids in postoperative inflammation using primary cultured rat intestinal smooth muscle (RISM) cells. Our studies indicate that S1P induces an appreciable proinflammatory response in these cells by elevating the cyclooxygenase-2 (COX-2) protein expression and formation of prostaglandin E₂ (PGE₂), the most abundant COX-2 product. S1P-induced COX-2 expression was strongly inhibited by pertussis-toxin (PTX), indicating the involvement of G_{i/o} protein coupled S1P receptors. Studies with pharmacological inhibitors indicate that S1P-induced COX-2 expression is mediated by both, extracellular-signal regulated kinase (ERK) and p38 mitogenic-activated protein kinase (p38 MAPK). In addition, an increased secretion of interleukin-6 (IL-6), after S1P administration was observed. We have also shown that S1P is involved in tumor necrosis factor- α (TNF- α) induced IL-6 secretion. Indeed, an increased sphingosine kinase (SK) activity was observed upon stimulation of RISM cells with TNF- α . C1P also enhanced PGE₂ synthesis but without any effect on COX-2 expression. However, C1P stimulated arachidonic acid release, which is indicative for the activation of phospholipase A₂ (PLA₂). Moreover, a direct correlation between mechanical stress and activation of S1P and C1P formation was established in RISM cells. Collectively, our results demonstrate that intestinal smooth muscle cells represent a major target for both, C1P and S1P activity. Thus, the elevated content of the two bioactive sphingolipids in this tissue could at least in part explain postoperative intestinal dysmotility.

In addition, in the present study the role of sphingolipids in lipopolysaccharide (LPS)-induced inflammatory reactions was addressed using a well-established sepsis model. We found an increase in the content of S1P and C1P after LPS administration indicating for the first time a direct involvement of these two bioactive sphingolipids in LPS signalling in an animal model. Moreover, studies on RISM cells reveal that SK mediates LPS-induced IL-6 secretion with a possible activation of ceramide kinase (CK).

The findings that the two bioactive sphingolipids specifically increase in the muscle layer after intestinal manipulation and upon LPS administration, as well as the fact that they induce and mediate pro-inflammatory responses in RISM cells, make them interesting targets for development of novel anti-inflammatory drugs. Our data raise the possibility to overcome postoperative- or sepsis-induced ileus by a targeted disruption of the sphingolipid-mediated inflammatory pathway.

1. Introduction

1.1. The intestine

The intestine, named “the inner tube of life” in a special issue of the Science magazine, is a huge tube, in human adult about 10 meter-long, and homes about 10 times more microorganisms than human cells [Simpson et al., 2005]. Scientists consider the gut as “the second brain” due to the presence of enteric nervous system, embedded into the wall of the intestine. The intestine is divided into two major sections: small intestine and large intestine.

1.1.1. Anatomy and function of the small intestine

The small intestine is a convoluted tube, extending from the pylorus to the large intestine. It is contained in the central and lower part of the abdominal cavity and is surrounded above and at the sides by the large intestine. It is connected to the vertebral column by a fold of peritoneum, the mesentery. The small intestine is divided into three structural parts: duodenum, jejunum and ileum. Food from the stomach is allowed into the duodenum by a muscle called pylorus or pyloric sphincter and is then pushed through the small intestine by a process of muscular contraction called peristalsis. The wall of the small intestine is composed of four layers: *tunica serosa*, *tunica muscularis*, *tela submucosa* and *tunica mucosa* (Fig. 1).

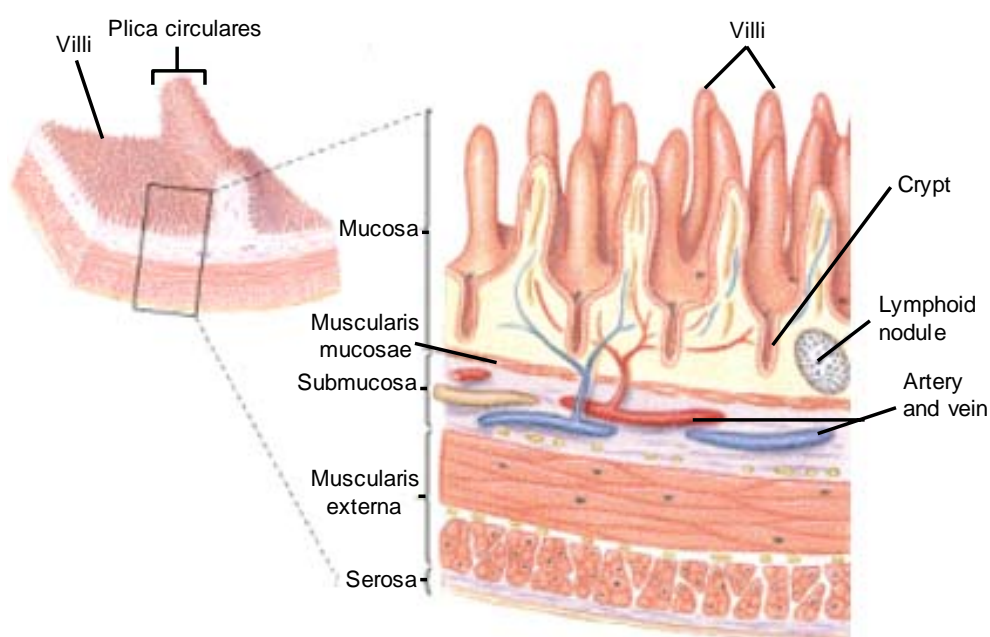


Fig. 1 Anatomy of the small intestinal wall.

<http://fire.biol.wvu.edu/lapsansk/348/DigestiveF05.ppt>

Tunica muscularis consists of two layers: an external longitudinal, and an internal, circular layer responsible for the peristaltic movements of the whole intestine. Numerous cellular populations inhabit the intestinal *muscularis* such as smooth muscle, enteric neurons, endothelium, macrophages etc. *Tela submucosa* connects together the *mucosa* and *muscularis* layers. It consists of connective tissue containing blood vessels, lymphatic vessels and nerves and it is the strongest layer of the intestine. There is a band of smooth muscles so called *muscularis mucosa* separating mucosa from submucosa. *Tunica mucosa* is highly modified and organized into circular folds (plica circulares) that contain a core of submucosa. Several histologically distinct cell types exist in the epithelial layer of *mucosa*, among which the enterocytes are the main type. The epithelium, which is lining the mucosa, forms villi, finger-like projections, each of which is surrounded by several very narrow pits, referred to as crypts (Fig.1). Within each villus, there is a layer of loose connective tissue called *lamina propria*, which contains blood and lymphatic capillaries, nerves, lymphatic nodules and aggregated nodules (Peyer's patches) and intestinal glands.

The main physiological functions of the small intestine are digestion and absorption processes [Schiller and Feldman, 1997]. The duodenum is largely responsible for digestion of food macromolecules into their low components with the assistance of pancreatic enzymes and bile salts, whereas jejunum and ileum are mainly responsible for absorption of nutrients from the lumen into the blood stream.

1.1.2. Postoperative ileus

The functions of the intestine can be altered by many internal or external factors. For example, surgical procedures in the abdomen inhibit bowel motility, leading to a condition known as postoperative ileus.

The term, "ileus" comes from the Greek word *eilo* that means to roll up tight. Postoperative ileus is a transient impairment of bowel motility that occurs after surgery and is usually considered an inevitable response to surgery. It is a significant clinical problem after surgical trauma and constitutes the most common reason for delayed discharge from the hospital after abdominal surgery. The prolonged dysfunction of bowel motility lasts for days with accumulation of secretions and gas, abdominal distension, nausea, vomiting, discomfort, pain and respiratory abnormalities including aspiration of gastrointestinal fluids. The economic impact of ileus has been estimated at 1 billion dollars per year in the United States [Livingston and Passaro, 1990]. Multiple causes of postoperative ileus have been suggested including the involvement of sympathetic reflexes, inhibitory humoral agents, anaesthetic agents and also

inflammatory events [Luckey et al., 2003]. The hypothesis that postsurgical ileus is accompanied by a scenario of inflammatory events is supported by studies using a simple rodent model of intestinal manipulation. It was shown that the simple abdominal surgery leads to activation of resident muscularis macrophages and a subsequent upregulation of cytokines, chemokines and kinetically active substances (nitric oxide, prostaglandins) [Kalff et al., 1998; Kalff et al., 2003]. This local inflammatory milieu then participates in the recruitment of leukocytes into the *muscularis* with a significant decrease in muscle contractile activity [Kalff et al., 1999a; Kalff et al., 1999b].

Although postoperative dysmotility has been documented by numerous investigators [Baker and Webster, 1968; Dahlgren and Selking, 1972; Graber et al., 1982; Condon et al., 1986; Schippers et al., 1991], the exact underlying mechanisms of this important clinical problem are still ill defined. To date, no accepted pharmacological prevention or treatment of postoperative ileus exists. Therefore, the elucidation of pathological events at the molecular and cellular level could help in finding new therapeutic strategies for prevention and/or treatment of this surgical complication.

1.1.3. Sepsis-induced ileus

The intestinal muscle dysfunction also occurs during sepsis. Sepsis (in Greek *Σήψις*, putrefaction) is a systemic inflammatory response to severe bacterial infection. It is a leading cause of morbidity and mortality in severely ill patients with about 9.3% of total deaths in the United States [Angus et al., 2001]. Sepsis frequently occurs after abdominal surgery, hemorrhagic shock, burns or trauma due to intestinal barrier failure and bacteria or endotoxin (lipopolysaccharide) translocation [Pape et al., 1994; Saadia, 1995; Deitch et al., 1996; Lichtman, 2001].

Lipopolysaccharide (LPS) often called endotoxin is an important structural component of the outer leaflet of the outer membrane of gram-negative bacteria such as *Escherichia coli*, *Salmonella*, *Pseudomonas* and others [Beutler and Rietschel, 2003] (Fig. 2). This glycolipid is the main constituent that is recognized by the innate immune system, the first line of defence against infection. Chemical analyses showed that LPS consists of a hydrophobic domain (lipid A), the toxic component of LPS that anchors the molecule in the outer membrane and a polysaccharide side chain, the non-toxic but immunogenic portion of LPS.

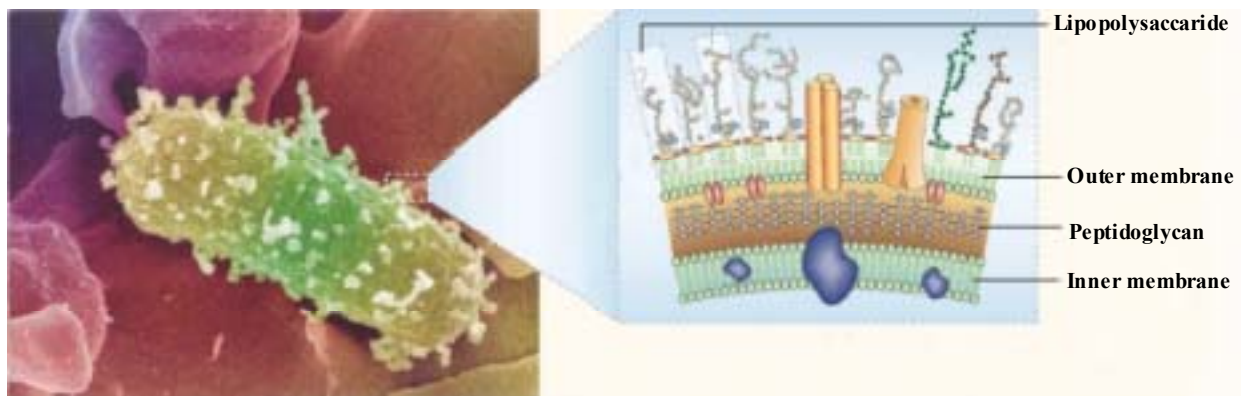


Fig. 2 A gram-negative bacterium. Electron micrograph of *Escherichia coli* with a schematic representation of LPS in the bacterial cell wall [Beutler and Rietschel, 2003].

During gram-negative infection, LPS interacts through its lipid A, with Toll-like receptor 4 (TLR4) (a homologue of *Drosophila* receptor Toll) present on the cell surface of monocytes, macrophages and non-hematopoietic cells [Akira and Takeda, 2004; Hirata et al., 2005; Singh and Schwartz, 2005; Rumio et al., 2006]. As a consequence, LPS activates several intracellular signalling pathways resulting in the activation of different transcription factors (NF- κ B, AP-1 Fos, Jun). Subsequently, these initiate the transcription of many genes necessary for the effector functions of innate immunity such as cytokines and adhesion molecules [Ulevitch, 1999; Alexander and Rietschel, 2001]. These physiological changes usually constitute a beneficial response for the affected host. However, in the case of bacterial sepsis an overwhelming production of proinflammatory cytokines such as TNF- α , IL-1 occurs. The overproduction of cytokines causes abnormal body temperature and respiratory rate, a drop in blood pressure and other devastating pathophysiological effects that ultimately lead to hemodynamic shock and multiple organ failure [Dinarello and Bunn, 1997; Calandra, 2001].

The intestine is not only the primary source of bacteria and bacterial toxins but is also a major target of bacterial-induced organ dysfunction. It is known that LPS from gram-negative bacteria is a causative factor of sepsis-induced ileus [Cullen et al., 1999a; Lodato et al., 1999]. It has been shown that the induction of sepsis by administration of living bacteria or endotoxin initiates an inflammatory cascade within the intestinal smooth muscle layer and a subsequent decrease in smooth muscle contractility [Eskandari et al., 1997].

As with postoperative ileus, there is no accepted pharmacological prevention or treatment of sepsis-induced ileus. Therefore, insights gained by studying sepsis-induced muscularis inflammatory events maybe helpful to develop an effective treatment strategy.

1.2. Inflammation

Inflammation is a defensive response that begins after cellular injury, which may be caused by microbes, physical agents (burns, radiation, trauma), chemicals (toxins, caustic substances), necrotic tissue and/or immunological reaction. As an indicator of disease, inflammation has been recognized for centuries. Almost 2000 years ago, the Roman physician, Celsus, described the four cardinal signs (symptoms) of inflammation: *rubor et tumor cum calore et dolore* (redness and swelling with heat and pain). Later on, another highlight pioneer in the history of inflammation research, Virchow, described the fifth cardinal sign of inflammation, *function laesa* (loss of function). The acute inflammatory response is characterized by a series of events including an increase in blood flow and vascular permeability and the accumulation of various inflammatory cells (neutrophils, monocytes, dendritic cells, mast cells and lymphocytes) at the site of tissue trauma. These phagocytic cells ingest foreign material and cell debris. Finally, the injurious stimulus is cleaned and normal tissue structure and function is restored. However, if an acute response is not resolved it becomes chronic. Prolonged inflammation is a hallmark of many chronic diseases and autoimmunity. Several types of mediators released or synthesized after target cell activation are known to play a role in the inflammatory response such as vasoactive amines (histamine, bradikinin), leukotrienes, prostaglandins and various cytokines and chemokines. Recent studies have demonstrated the activity of various sphingolipid molecules as potent mediators of the inflammatory pathway. Inflammatory responses in the bowel wall contribute, at least in part to postoperative ileus. Although the inflammatory mediators participating in postoperative ileus have not been well defined, production of prostaglandins and induction of IL-6 seem to be an important cause of postoperative ileus in rats [Schwarz et al., 2001; Wehner et al., 2005]

1.2.1. Prostaglandin generation: phospholipase A₂ enzymes and cyclooxygenases

Prostaglandins belong to a class of lipid mediators known as eicosanoids (from the Greek *eicosa*=twenty; for twenty carbon fatty acid derivatives) that are involved in various homeostatic biological functions and inflammation [Funk, 2001]. Prostaglandins are produced by most cells in our body and act as autocrine or paracrine lipid mediators. They are synthesized from membrane-released arachidonic acid when cells are activated by mechanical trauma, cytokines, growth factors, LPS or other stimuli [Scholz, 2003]. Prostaglandin biosynthesis is regulated by three enzymatic reactions. First, arachidonic acid, which is stored at the sn-2 position of the membrane glycerophospholipids, is released by the hydrolytic

action of phospholipase A₂ (PLA₂) of which several isoenzymes are known. Further on, arachidonic acid is metabolized to prostaglandin H₂ (PGH₂) by cyclooxygenase (COX) (1 and 2) enzymes also known as prostaglandin H synthases (PGHS). COX enzymes catalyze both: i) the bis-oxygenation of arachidonic acid leading to production of PGG₂ (COX reaction) and ii) reduction of 15-hydroperoxide of PGG₂ leading to formation of PGH₂ (peroxidase reaction). PGH₂ is subsequently converted to the most important biologically active end products such as PGD₂, PGE₂, PGF_{2α}, PGI₂ (prostacyclin) or TXA₂ (thromboxane) by specific cellular synthases (Fig. 3).

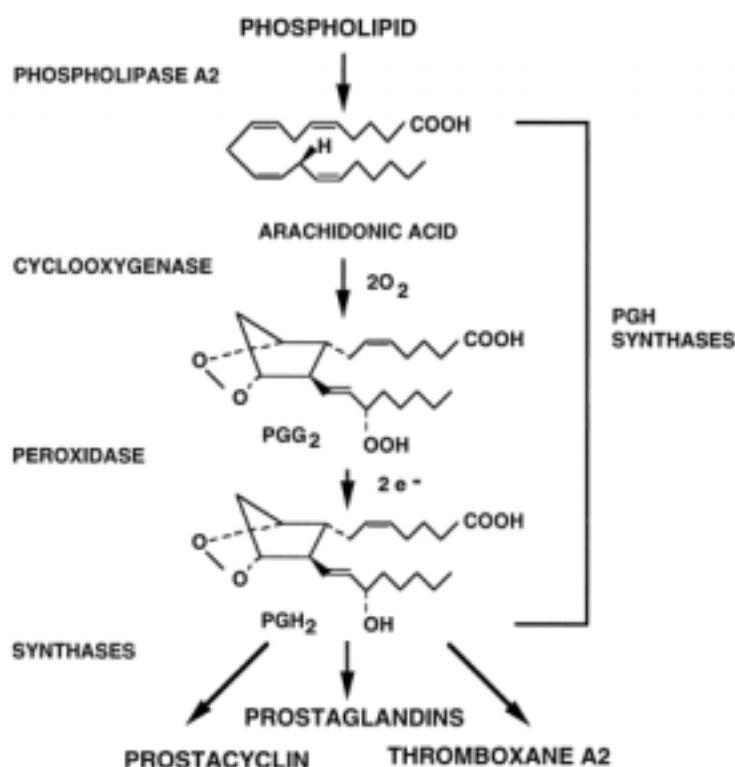


Fig. 3 Prostanoid biosynthetic pathway. In response to extracellular stimuli such as a cytokine, a growth factor or a circulating hormone, phospholipases are activated and arachidonic acid is hydrolyzed from the *sn*-2 position of membrane phospholipids. Arachidonate is converted by PGHS to PGH₂, and then PGH₂ is isomerised to biological active prostanoid products [Smith et al., 1996].

Once formed, the prostaglandins are released from the cells through a known prostaglandin transporter (PGT) [Schuster, 1998] and act via specific cell surface G-protein coupled receptors (EP₁₋₄, DP₁₋₂, FP, IP, TP_{α-β}), thus exerting their biological function [Hirai et al., 2001; Narumiya and FitzGerald, 2001; Simmons et al., 2004]. Of these, PGE₂ is the most common prostanoid, being produced by a wide variety of cells and tissues and playing crucial roles in various biological events. Moreover, PGE₂ is generated in substantial amounts at the site of inflammation and can mediate many of the pathological features of inflammation,

including vasodilation, febrile responses and platelet aggregation [Williams and Peck, 1977; Dinarello, 1984; Ballou et al., 1992]. The regulation of the prostaglandin biosynthetic pathway has become more complex with the discovery of multiple PLA₂ and COX enzyme forms.

1.2.1.1. Phospholipase A₂ enzymes

Phospholipase A₂ (PLA₂) enzymes hydrolyse the sn-2 ester bond of phospholipids to release free fatty acids and lysophospholipids. Depending on the Ca²⁺ requirement for their enzymatic activity, PLA₂ can be classified in three categories: secretory PLA₂ (sPLA₂) that require millimolar concentrations of Ca²⁺, the cytosolic PLA₂ (cPLA₂) that require micromolar concentration of Ca²⁺ and the Ca²⁺-independent PLA₂ (iPLA₂) that do not require Ca²⁺ for activity [Akiba et al., 2004]. Of these, cPLA₂ has been shown to play a pivotal role in biosynthesis of inflammatory lipid mediators (prostaglandins, leukotrienes) since it is highly selective for glycerophospholipids containing arachidonic acid in the sn-2 position [Shimizu and Wolfe, 1990; Clark et al., 1995; Hirabayashi et al., 2004]. While α-, β-, γ-isoforms of cPLA₂ exist, cPLA₂α appears to be most relevant for prostaglandin formation during acute and chronic inflammation. cPLA₂α is comprised of two catalytic domains A and B and a C2 domain also known as calcium-lipid binding domain or CaLB domain. cPLA₂α does not require calcium for catalysis but for binding to the membrane, this association being stabilized by prolonged calcium elevation that causes an abundant arachidonic acid release. cPLA₂α can be activated by mechanical trauma, cytokines, LPS, hormones and antigens. This triggers translocation of PLA₂α to endoplasmatic reticulum (ER), Golgi apparatus and nuclear membranes where it induces together with COX enzymes PGE₂ formation. Studies with cPLA₂α-deficient mice have implicated this enzyme in many pathological conditions, especially those which are associated with inflammation [Bonventre et al., 1997; Nagase et al., 2000; Takaku et al., 2000; Miyaura et al., 2003; Kalyvas and David, 2004].

1.2.1.2. Cyclooxygenases

In 1990 it was established that COX, the enzyme that catalyses the conversion of arachidonic acid to prostaglandins, exists in two isoforms, commonly referred to as COX-1 and COX-2 [Xie et al., 1991; Vane et al., 1994]. They are membrane-bound proteins, COX-1 residing primarily in the ER, whereas COX-2 is located predominantly in the perinuclear envelope. Recent studies suggested the existence of another COX enzyme, namely COX-3, a splicing variant of COX-1, which is known to be a 5.2-kb transcript most abundant in the cerebral

cortex and heart [Chandrasekharan et al., 2002]. COX-1 is expressed constitutively in most tissues and cell types. Therefore, COX-1 is considered to exert “housekeeping” functions such as maintenance of vascular tone and mucosal integrity in the gastrointestinal tract [Adami et al., 2006]. Unlike COX-1, COX-2 is the inducible isoform whose expression is highly up-regulated in many cell types in response to trauma, proinflammatory agents such as IL-1, TNF, LPS as well as by growth factors [Raz et al., 1989; Ferraz et al., 1997]. Thus, COX-2 is mainly considered to mediate inflammation. COX-2 has emerged as the isoform responsible for the synthesis of eicosanoids in pathological processes such as acute and chronic inflammation, neurodegenerative diseases including amyotrophic lateral sclerosis and Alzheimer’s disease as well as in heart diseases [Turini and DuBois, 2002]. In addition, COX-2 is also found to be overexpressed in transformed cells and in malignant tissue [Eberhart et al., 1994; Kargman et al., 1995; Ristimaki et al., 1997]. Importantly, COX-2 null mice were protected against the development of both intestinal and skin tumors [Oshima et al., 1996; Morham et al., 1997]. Both isoforms are inhibited by glucocorticoids and non-steroid anti-inflammatory drugs (NSAIDs) like aspirin [Hinz and Brune, 2002]. However, NSAIDs administration is accompanied by many side effects including gastrointestinal ulceration, bleeding, and platelet dysfunctions due to suppression of COX-1 derived-prostaglandins, which are required for normal homeostatic processes. Therefore, during the last years much research has been done to develop potent and selective COX-2 inhibitors able to limit inflammation without adverse effects [Kawamori et al., 1998; Brune and Hinz, 2004].

1.2.2. Cytokine signalling: tumor necrosis factor- α and the interleukins

Cytokines represent another important class of mediators involved in induction of the inflammatory response and in immunity [Sartor, 1994; Borish and Steinke, 2003]. They are low molecular weight proteins or glycoproteins secreted by most cells of the immune system as well as by other cells in the body in response to a number of inducing stimuli. The cytokine family includes: interleukins (IL), interferons (IFN), tumor necrosis factors (TNF), transforming growth factor β (TGF- β), and chemokines. Although most cytokines exert pro-inflammatory effects through binding to specific cytokine receptors present on their target cells, some of these cytokines elicit anti-inflammatory functions such as, IL-1ra, IL-10, IL-13, TGF- β [Del Prete et al., 1993; Muchamuel et al., 1997; Arend and Guthridge, 2000; Wahl and Chen, 2005]. Therefore, it is important to study the levels and the action of these molecules in order to understand the mechanisms of inflammatory states. Among many cytokines described so far, three in particular, namely, TNF- α , IL-1 β and IL-6 attracted significant

attention due to their role in the pathophysiology of many inflammatory responses as well as of septic shock [Fiocchi, 1998; Monack et al., 2004].

1.2.2.1. Tumor necrosis factor- α

TNF- α previously termed cachectin because of its possible role in cachexia or wasting effect [Beutler, 1989], belongs to the TNF family that includes 19 ligands [Aggarwal, 2003]. It is mainly produced by monocytes and macrophages but can be released also by other cells including, neutrophils, mast cells, lymphocytes and endothelial cells. TNF- α is produced in response to many extracellular stimuli such as cytokines, heat shock, UV irradiation and microbial infection. It is considered to be a primary mediator of immune regulation, sepsis shock and inflammatory response as well as an anti-tumor factor [Tracey and Cerami, 1993]. TNF- α has potent proinflammatory effects since it activates neutrophils, stimulates endothelial cells to produce adhesion molecules and increases vascular permeability facilitating migration of inflammatory cells to the sites of inflammation [Oppenheim, 2001; Borish and Steinke, 2003]. TNFs exert their effects by binding to specific cell surface receptors. There are two types of TNF receptors: TNFR1 expressed in almost all cell types and TNFR2 which seems to be highly regulated and expressed in immune and endothelial cells [Tartaglia and Goeddel, 1992]. TNFRs signal via distinct motifs in their intracytoplasmatic domains such as TNFR-associated factor (TRAF) and TNF-R-associated death domain protein (TRADD) [Arch et al., 1998]. These motifs associate with different adaptors and thus signal distinct cell functions [Clark et al., 2005]. TNFR1 appears to be primarily responsible for the proinflammatory effect of TNF- α under physiological conditions. For instance, TNFR1 can activate NF- κ B by recruiting TRADD, TRAF2 and receptor interacting protein (RIP) [Chen and Goeddel, 2002]. NF- κ B is a transcription factor that regulates the synthesis of many proteins that function in inflammatory pathways including, IL-1, IFN- β , TNF- α and cyclooxygenases [Ghosh et al., 1998].

1.2.2.2. Interleukin-1 β

IL-1 β , a member of IL-1 family is a key mediator of pro-inflammatory responses. It was originally described as the “endogenous pyrogen” because it has pronounced fever-induced properties in rabbits [Huisling et al., 2004]. IL-1 β is synthesized as a large precursor protein by many cell types including monocytes and lymphocytes. Pro-IL-1 β is biologically inactive and requires cleavage by caspase-1 to produce an active 17 kDa protein, which is secreted. IL-1 β exerts biological effects by binding to specific cell surface receptors [Subramaniam et al.,

2004]. It activates T-lymphocytes, B-cell proliferation and immunoglobulin production, stimulates synthesis of acute phase proteins and endothelial cell adherence of leukocytes through up-regulation of adherence molecules (ICAM-1, VCAM-1, E-selectin) [Oppenheim, 2001].

1.2.2.3. Interleukin-6

Interleukin-6 (IL-6) is a pleiotropic cytokine with a wide range of biological activities [Papanicolaou et al., 1998]. Similar to TNF- α , IL-6 is produced not only by immune cells but also by many nonimmune cells such as osteoblasts, keratynocytes, intestinal epithelial cells and smooth muscle cells [Heinrich et al., 1990; Loppnow and Libby, 1990; McGee et al., 1993]. It has potent proinflammatory effects including immunoglobulin production, T-cell activation and differentiation, induction of fever and synthesis of acute phase proteins such as C-reactive protein and complement components [Oppenheim, 2001]. A variety of molecules including IL-1, TNF- α , PDGF, IFN- β and calcium ionophore A23187 induce production of IL-6. On the other hand, IL-6 inhibits the secretion of IL-1 and TNF- α and induces the synthesis of IL-receptor antagonist (IL-Ra) and glucocorticoids, which are known for their anti-inflammatory actions suggesting that IL-6 has both, proinflammatory and anti-inflammatory properties [Libert et al., 1994; Xing et al., 1998]. In models of chronic inflammation, IL-6 has a proinflammatory effect that favours the recruitment of mononuclear cells such as macrophages and lymphocytes at the site of injury [Alonzi et al., 1998; Yamamoto et al., 2000], whereas in models of acute inflammation IL-6 exhibits an anti-inflammatory profile [Xing et al., 1998].

1.2.3. Sphingolipids

1.2.3.1. Sphingolipid metabolism and function. General remarks

In 1884, the German pathologist, J.L. W. Thudichum discovered and named a class of new lipids while studying the chemical composition of the brain. Hydrolysis of these lipids produced a compound, which Thudichum named “sphingosin” due to its enigmatic “Sphinx-like” properties [Thudichum, 1884]. Thus, this class of lipids became known as the *sphingolipids* because of their common sphingosine backbone. Sphingolipids are ubiquitous constituents of membranes in animals, plants, fungi, yeast and some prokaryotic organisms and they were originally regarded as structural and inert components of cell membranes. Currently, many studies support their roles in modulating various cellular events such as proliferation, differentiation, apoptosis and inflammation [Merrill et al., 1997; El Alwani et al., 2006]. Sphingolipid metabolism is a complex network of highly regulated pathways that produces bioactive molecules including primarily ceramide, ceramide-1-phosphate, sphingosine -1-phosphate, sphingosine and glucosylceramide (Fig. 4).

Sphingolipid biosynthesis starts at the cytoplasmic leaflet of the ER with the condensation of palmitoyl-CoA and L-serine yielding 3-ketosphinganine. The reaction is catalysed by a pyridoxal phosphate (PLP)-dependent enzyme serine palmitoyltransferase and represents the rate-limiting step of sphingolipid biosynthesis [Mandon et al., 1992]. The next step is the reduction of 3-ketosphinganine to *D-erythro*-sphinganine in the presence of NADPH. Subsequently, sphinganine is acylated to dihydroceramide by (dihydro) ceramide synthase. This enzyme is inhibited by a fungal toxin, fumonisin B1 [Vesper et al., 1999]. Insertion of the 4,5-*trans*-double bond into the sphingoid base backbone of dihydroceramide results in the formation of ceramide [Rother et al., 1992; Michel et al., 1997] (Fig. 4). Ceramide is the central lipid in the metabolism of sphingolipids [van Echten-Deckert and Herget, 2006]. Once formed, it serves as a precursor for more complex glycosphingolipids and sphingomyelin (SM). SM is synthesized by the transfer of phosphorylcholine from phosphatidylcholine to ceramide, liberating diacylglycerol through the action of sphingomyelin synthase [Ramstedt and Slotte, 2002]. Neutral glycosphingolipids like glucosylceramide (GlcCer) and galactosylceramide (GalCer) are generated by a direct transfer of a single sugar from the appropriate sugar nucleotide (UDP-Glc, UDP-Gal, etc) to ceramide by glycosyltransferases [van Echten-Deckert and Dragusin, *submitted*] Another important metabolite that can be generated from ceramide is ceramide-1-phosphate (C1P), which is formed by the action of ceramide kinase [Bajjalieh et al., 1989] (Fig. 4).

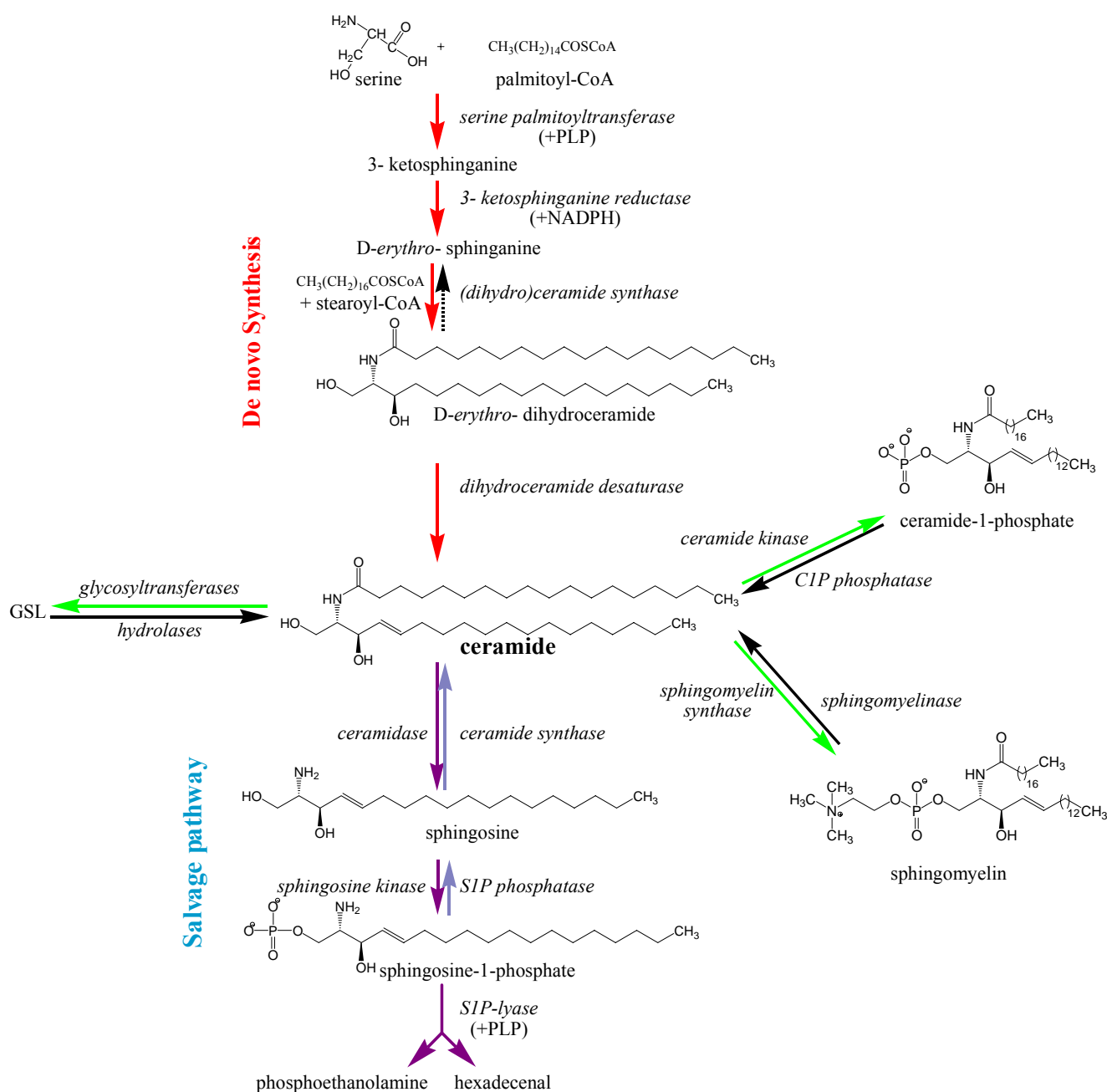


Fig. 4 Scheme of sphingolipid metabolism. Ceramide is the central molecule in the metabolism of sphingolipids. It can be formed *de novo* (red arrows), from hydrolysis of sphingomyelin or glycosphingolipids, or from dephosphorylation of ceramide-1-phosphate (C1P) (black arrows) or recycling of sphingosine (salvage pathway). Ceramide can serve as a precursor in many biosynthetic pathways (green arrows). The major pathway for catabolism of ceramide is its deacylation by ceramidases to sphingosine, which in turn is phosphorylated to generate sphingosine-1-phosphate (S1P) (dark blue arrows). S1P can be irreversibly cleaved by lyase to phosphoethanolamine and hexadecenal (dark blue arrows) or it can be dephosphorylated by phosphatases that regenerate sphingosine in the ceramide salvage pathway (light blue arrows). GSL, glycosphingolipids; PLP, pyridoxal phosphate; NADPH, nicotine adenine dinucleotide phosphate. [Wedeking and van Echten-Deckert, 2006]

Catabolism of sphingolipids occurs primarily in the lysosomal compartment through the action of several distinct hydrolases that sequentially remove the headgroups of complex sphingolipids, resulting in the formation of ceramide [Hannun et al., 2001]. Non-lysosomal

hydrolases also exist in other compartments including golgi apparatus and plasma membrane [Goni and Alonso, 2002]. SM and glycosphingolipids are degraded to ceramide through the action of sphingomyelinases (SMase) and exoglycosidases, respectively [Hannun, 1994; van Echten-Deckert and Herget, 2006]. The major pathway for catabolism of ceramide is its deacylation by ceramidases to sphingosine [Hassler and Bell, 1993]. In turn, sphingosine may be phosphorylated into sphingosine-1-phosphate (S1P) by the action of sphingosine kinase (SK), or acylated by ceramide synthase in the salvage pathway to form ceramide [Hannun et al., 2001] (Fig. 4). S1P can also enter the salvage pathway through the action of phosphatases that regenerate sphingosine, or it can be irreversibly cleaved to phosphoethanolamine and hexadecenal by a pyridoxal 5'-phosphate (PLP)-dependent lyase [Van Veldhoven, 2000; Ikeda et al., 2004] (Fig. 4). The aldehyde intermediate is oxidized to fatty acid whereas phosphoethanolamine can be utilized for the synthesis of phosphatidylethanolamine, both of which may enter glycerolipid pathway [Merrill and Sweeley, 1996].

Experimental evidence over the last decades supports the notion that sphingolipids are involved in a wide variety of biological processes. Together with cholesterol they form distinct microdomains in the plasma membranes that may serve primarily for cellular signal transduction [Szabo et al., 2004]. Complex glycosphingolipids enriched in the plasma membrane mediate interaction with the extracellular matrix and serve as attachment sites for microorganisms, microbial toxins and viruses [Holmgren et al., 1975; Markwell et al., 1981; Merrill and Sweeley, 1996]. Instead sphingomyelin influences membrane rigidity and stability [Ridgway, 2000]. In addition to their structural and regulatory roles, sphingolipids have been implicated in diverse processes including regulation of transformation, differentiation, proliferation, apoptosis and inflammation [Ogretmen and Hannun, 2004; Wedeking and van Echten-Deckert, 2006]. Among many sphingolipids with important biological roles, ceramide, sphingosine-1-phosphate and recently ceramide-1-phosphate received significant attention.

1.2.3.1.1. Ceramide

Ceramide has proven to be a powerful signalling molecule produced in response to various stimuli such as cytokines, heat, chemotherapeutic agents, UV irradiation, LPS and other agents [Obeid et al., 1993; Chang et al., 1995; Haimovitz-Friedman et al., 1997; Hannun and Obeid, 2002]. Ceramide, once generated, is a mediator of multiple cellular signalling pathways that involve cell cycle arrest [Jayadev et al., 1995], cell differentiation [Okazaki et al., 1989], apoptosis [Obeid et al., 1993], aging, [Venable et al., 1995], and other cell responses. Ceramide actions are mediated by regulation of intracellular effectors such as

ceramide-activated protein-phosphatases (CAPP), namely PP1A and PP2A [Chalfant et al., 1999] ceramide-activated protein kinase (CAPK) [Zhang et al., 1997], PKC ζ [Wang et al., 2005] and cathepsin D [Heinrich et al., 1999]. Ceramide is the common intermediate for S1P and C1P.

1.2.3.1.2. Sphingosine-1-phosphate

Unlike ceramide, which has been associated with growth arrest and cell death, sphingosine-1-phosphate (S1P) enhances growth and survival in diverse cell types [Spiegel and Milstien, 2002]. S1P is generated from sphingosine through the action of two SKs: SK1 and SK2 [Kohama et al., 1998; Liu et al., 2000a]. Although both SKs are closely related and phosphorylate sphingosine to produce S1P, they show different effects on cell survival and sphingolipid metabolism [Maceyka et al., 2005]. In contrast to other sphingolipid second messengers, S1P might have dual functions. First, S1P was found to act as an extracellular ligand by binding to a family of five specific G-protein coupled receptors (GPCR), originally named endothelial differentiation gene receptors (EDG-1,-3,-5,-6,-8) [Lee et al., 1998] and now termed S1P₁₋₅ receptors [Spiegel and Milstien, 2003] (Fig. 5). These receptors are ubiquitously expressed and coupled to various heterotrimeric G proteins leading to activation of numerous downstream signalling pathways. Consequently, S1P has the ability to regulate various physiological processes including angiogenesis and vascular maturation [Wang et al., 1999; Liu et al., 2000b], heart development [Kupperman et al., 2000] and immunity [Graeler et al., 2002; Mandala et al., 2002], depending on the tissue-specific expression of S1P receptors and the respective coupled G proteins.

Later on, it has been suggested that S1P also functions as an intracellular second messenger generated in response to a variety of external stimuli in particularly growth and survival factors including platelet-derived growth factor (PDGF) [Olivera and Spiegel, 1993], epidermal growth factor, (TNF- α) [Xia et al., 2002], nerve growth factor [Edsall et al., 1997], S1P itself [Meyer zu Heringdorf et al., 2001] and others, which activate SK to generate S1P [Payne et al., 2002] (Fig. 5). Intracellular S1P has been found to play a key role in calcium mobilization, cell growth and survival [Olivera et al., 1999]. Extracellular and intracellular actions of S1P are mediated by activation of different MAPKs [Gonda et al., 1999; Shu et al., 2002].

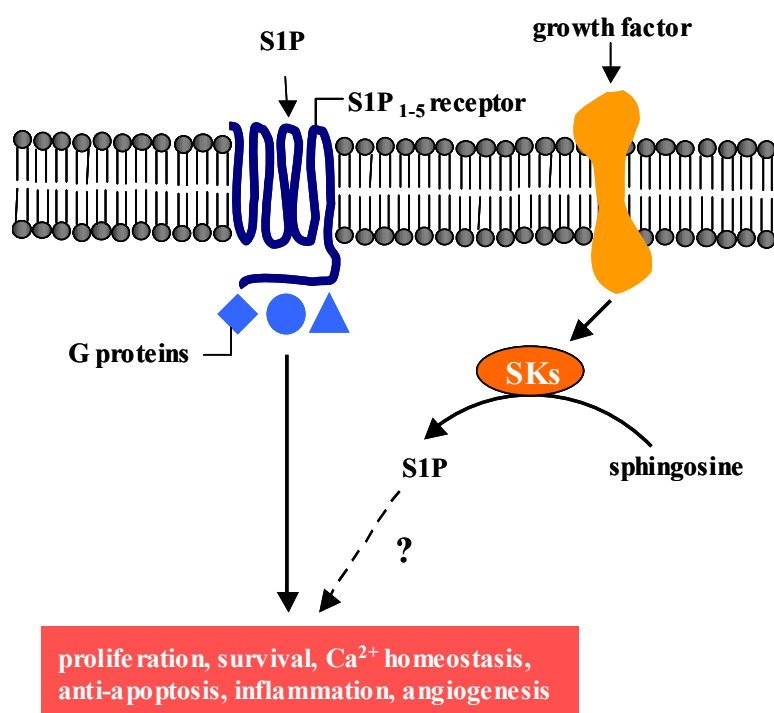


Fig. 5 Dual function of sphingosine-1-phosphate. S1P can act as a second messenger or as an extracellular ligand. Cellular S1P is produced in response to diverse agonists such as, growth factors or cytokines that bind to cell surface receptors and activate sphingosine kinases (SKs). SKs phosphorylate sphingosine leading to increased generation of S1P. S1P can also function as a specific ligand of G-protein-coupled S1P receptors. S1P receptors couple with various G proteins such that S1P can mediate distinct biological processes.

1.2.3.1.3. Ceramide-1-phosphate

Another bioactive phosphosphingolipid is ceramide-1-phosphate (C1P), which is produced in mammalian cells by phosphorylation of ceramide by ceramide kinase. C1P can also be generated from sphingomyelin by sphingomyelinase D (SMase D), the main component of the venom from *Loxosceles reclusus* (brown recluse spider) [Kurpiewski et al., 1981; Merchant et al., 1998]. However, a mammalian SMase D has not been described so far. Although C1P was identified more than a decade ago, its potential biological functions have only become clear in the last few years. The existence of C1P was first reported in human leukaemia (HL-60) cells [Dressler and Kolesnick, 1990]. The first data regarding C1P activity indicated that it stimulates DNA synthesis and promotes cell division in rat fibroblasts [Gomez-Munoz et al., 1995]. Other important findings implicated C1P in phagocytosis by promoting phagolysosome formation [Hinkovska-Galcheva et al., 1998], in blocking apoptosis through inhibition of acidic SMase in macrophages [Gomez-Munoz et al., 2004] and in mast cell degranulation in a calcium-dependent manner [Mitsutake et al., 2004]. Unlike S1P, C1P does not act through a cell surface receptor. Recent studies suggest an intracellular role for C1P as second messenger that involves C1P in inflammatory responses [Pettus et al., 2004a].

1.2.4. MAPKs in inflammatory responses

Considerable attention has also been given to mitogen-activated protein kinases (MAPKs) as possible targets for development of novel anti-inflammatory therapeutics. Inhibitors targeting these pathways have been developed and preliminary data suggest that they indeed exhibit anti-inflammatory activity.

The MAPK cascade is one of the most ancient and evolutionarily conserved signalling pathways, which is also important for many physiological processes [Seger and Krebs, 1995]. MAPK pathways can be activated by a wide variety of different stimuli including hormones and growth factors, cytokine, environmental stresses such as osmotic shock, ionizing radiation and ischemic injury. In turn, MAPK signalling pathway coordinates activation of gene transcription, protein synthesis, cell cycle machinery, cell death and differentiation [Herskowitz, 1995; Marshall, 1995; Kyriakis and Avruch, 1996]. The first enzyme that is activated in MAPK cascade is a Ser/Thr kinase known as MAPK kinase kinase (MAPKKK). These MAPKKKs phosphorylate and activate MAPK kinases (MKK), which are dual-specificity kinases catalysing the phosphorylation of Tyr or Ser/Thr residues in its target proteins. Active MKK activates MAPK through phosphorylation of both, a Thr and a Tyr, which are separated in the protein by a single amino acid [Garrington and Johnson, 1999; Chang and Karin, 2001]. MAPKs are also Ser/Thr kinases that phosphorylate many different proteins including nuclear transcription factors and other protein kinases that mediate cellular processes (Fig. 6). MAPK-catalysed phosphorylation of substrate proteins can turn on or off the activity of the respective target. MAPKs are inactivated by MAPK phosphatases [Farooq and Zhou, 2004].

There are three well-characterized subfamilies of MAPKs in mammalian cells. These MAPKs include the extracellular signal-regulated kinases (ERK) [Schaeffer and Weber, 1999], the p38 MAP kinases [Ono and Han, 2000], and the c-Jun NH₂-terminal kinases (JNK) [Davis, 2000]. ERK pathway was identified downstream of oncogene Ras and is involved in the regulation of cell growth and differentiation. There are two isoforms of ERK, ERK1 and 2, which are sometimes referred to as p44/42 MAPK. They are widely expressed and activated by many different stimuli, including growth factors, cytokines, virus infection, ligands for G-protein coupled receptors (GPCRs) [Ashwell, 2006].

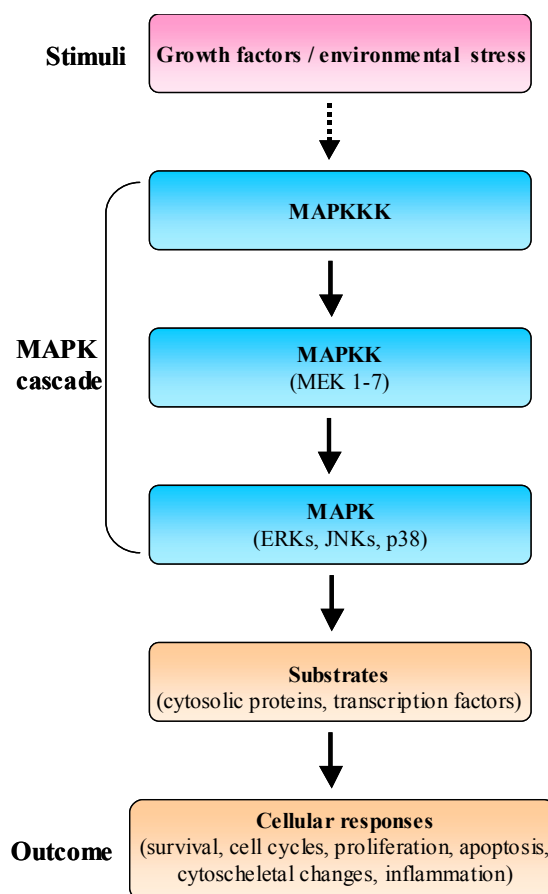


Fig. 6 MAP kinase pathways. Mitogen-activated protein kinases (MAPKs), which integrate and process various extracellular signals. The MAPK cascade consists of a series of three protein kinases — a MAPK and two upstream components, MAPK kinase (MAPKK or MEK) and MAPKK kinase (MAPKKK). ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase [Kumar et al., 2003].

There is relative little information on the role of the ERK cascade in inflammation. However, it was reported that LPS-induced TNF- α production is regulated via an ERK-dependent pathway [Dumitru et al., 2000]. Although no direct ERK inhibitors have been reported yet, several inhibitors that interfere with the activity of MKKs that act upstream of ERK have been described. These compounds including PD 98059 [Dudley et al., 1995] and U0126 [Duncia et al., 1998] that are quite effective in inhibiting ERK activation.

There are four p38 MAPK isoforms, α , β , γ and δ respectively, each encoded by a separate gene. The p38 α enzyme is the best characterized and is expressed in most cell types. p38 MAPK was first identified as an IL-1 and LPS activated kinase [Freshney et al., 1994; Han et al., 1994]. Therefore, from the very beginning it was expected to play an important role in inflammation. Other data that support the role of p38 in inflammatory responses were derived from studies with p38 inhibitors such as SB 203580 and SB 239063, which potently inhibited the production of proinflammatory cytokines in different cell types [Cuenda et al., 1995; Underwood et al., 2000; Barone et al., 2001]. Moreover, administration of SB 203580 was

found to have beneficial effects in animal disease models such as collagen-induced arthritis and endotoxin induced sepsis-shock [Lee et al., 1999]. p38 MAPKs are also activated by many other stimuli including hormones, ligands for GPCRs and stresses such as osmotic shock and heat shock [Johnson and Lapadat, 2002].

JNKs (JNK 1, 2 and 3) were first isolated and characterized as stress-activated protein kinases (SAPKs) due to their activation in response to inhibition of protein synthesis. JNK activity can be strongly induced in multiple cell types by proinflammatory stimuli such as LPS, TNF- α and IL-1 [Davis, 2000; Chang and Karin, 2001]. It was reported that JNKs bind, phosphorylate and increase transcription activity of the DNA binding protein c-Jun. c-Jun is a component of the activating protein 1 (AP-1) transcription complex, an important regulator of gene transcription. AP-1 and related transcription factors such as the activating transcription factor 2 (ATF-2) play an important role in inflammatory response due to their ability to contribute to the induction of cytokine genes such as those that encode for TNF- α , IFN β and IL-6 [Karin, 1995; Chu et al., 1999]. Several different JNK inhibitors have been recently identified. One of them, namely SP6000125 has been tested in a rat model of rheumatoid arthritis and it was found effective not only in reducing inflammation but also in prevention of tissue damage [Han et al., 2001].

1.3. Aim of the study

Several studies suggest a role of sphingolipids in inflammatory responses. However, data regarding the possible proinflammatory effects of sphingolipids in a pathological state are not available yet.

A local inflammatory reaction within the intestinal *muscularis* has been proven to be causative for postoperative ileus in both, animal model and human situation. The aim of the present study was to investigate the potential involvement of sphingolipids in inflammatory processes known to accompany postoperative- and sepsis-induced ileus.

The following questions arise from this aim:

- 1) Does intestinal manipulation or LPS administration alter sphingolipid composition in rat small intestine?
- 2) Are sphingolipids able to induce an inflammatory response in primary cultured rat intestinal smooth muscle cells (RISM cells), the prevailing cell type in tunica *muscularis*?
- 3) Which is the molecular mechanism underlying the proinflammatory action of sphingolipids?

2. Materials and Methods

2.1. Materials

Apparatus

Apparatuses	Supplier	Location
Agarose gel electrophoresis	ComPhor Midi; Biozym	Oldendorf
Analytical balance	BP 210 D; Sartorius	Göttingen
Autoclave	Fedegari Varioclave 50050; H+P Labortechnik	Albuzzano; Italien Oberschlesien
Balance	Sartorius	Göttingen
Bench centrifuge	Centrifuge 5417R; Eppendorf	Hamburg
Blotting Apparatus	Mini TransBlot; BioRad	München
Bioflex collagen 1 culture plate		
Centrifuge	Megafuge 2.0 R; Heraeus Multifuge 3 S-R; Heraeus	Hanau
Deep freezer (+4°C, -20°C)	Robert Bosch GmbH	Geringen- Schillerhöhe
Deep freezer (-80°C)	NapCoil UF 5000; NAPCO	Winchester, USA
Dish washer	Mielabor G7783; Miele	Köln
Drying cupboard	Heraeus	Hanau
ELISA-Reader	Multiscan Ascent, Dynex Hybaid	Frankfurt
Fluorimeter	Digitalfluorimeter Modell 8-9; Locarte, Dynex Hybaid Labsystems	Frankfurt
Gel apparatus for SDS-PAGE	Minigel; Biometra Multigel-Long; Biometra	Göttingen
Gel analysing system	AlphaDigiDoc; Biozym	Oldendorf
Heating-agitator	Ikamag-Ret; Ika-Labortechnik	Staufen
Imager-Plates (Screens)	BAS MS 2040 Imaging Plate Raytest	Straubenhardt
Incubators	Heraeus	Hanau
Incubation shaker	KS-250 basic; Ika-Labortechnik	Staufen
Laminar flow	Biohazard; Gelaire Heraeus	Mailand, Italien Hanau
Microwave	Privileg; ProMarkt	Bonn

Mixer-mill	Retsch	Haan
Peristaltic pump	P1; Pharmacia-LKB	Freiburg
Phospho Imager	Fujix BAS 1000; Raytest	Straubenhardt
Photometer	Smart Spec 3000; BioRad	München
Pipettes	Eppendorf Research 0,5-10, 10-100, 1-1000; Eppendorf	Hamburg
pH-Meter	PH 537; WTW	Weilheim
Reacti-Therm III	Pierce Chemical Company	Rockford, USA
Rotator	Stuart	SUA
Röntgen cassette	Chronex; DuPont	de Nemour, France
Scintillation counter	Packard Tricarb 1600 TR	Rodgau-Jügesheim
Shaking water bath	1083; Gesellschaft für Labortechnik	Burgwedel
Sonicator	Sonorex RK 100, Bandelin	Berlin
Thermocycler	PTC-200; MJResearch/Biozym	Oldendorf
Thermomixer	Comfort; Eppendorf	Hamburg
Ultrasonicator	Sonifer B12 with water cooled cup-horn; Branson Sonic Power Company	Danbury, SUA
Vortexer	Bender-Hohlbein	Zürich, Schweiz
Voltage supply source	Consort; Biometra PowerPac 3000; BioRad	Göttingen München
Water filtration apparatus	EasypureUV/UF; Barnstedt/Werner MembraPure; Millipore	Leverkusen Schwalbach

Expendable materials

Items	Supplier	Location
Cell culture flasks	Costar	Cambridge, MA/USA
Cell scrapers	Costar	Cambridge, MA/USA
Centrifuge tubes, 15 ml	Greiner	Nürtingen
Centrifuge tubes, 50 ml	Falcon; Beckton-Dickinson	Heidelberg
DC-Plates Kieselgel 60	Merck	Darmstadt
Disposable Filter Unit	Whatman	Dassel
Epis, 1,5/2 ml	Eppendorf	Hamburg
Filter paper	Macherey-Nagel	Düren
Homo-polymer microtubes, 1.5 ml, MCT-150-C	Axygen Scientific	Union City, CA, USA

Microtiter plates	Falcon; Beckton-Dickinson	Heidelberg
Nitrocellulose-Membranes	Porablot; Macherey-Nagel	Düren
Parafilm	AAC	Greenwich, USA
Petri dishes (8 cm ² , 21cm ²)	Falcon; Beckton-Dickinson	Heidelberg
Pipette tips	Greiner	Nürtingen
Pyrex tubes	VWR	Darmstadt
Röntgen films	Kodak Biomax MS-1; Sigma	Taufkirchen
Scintillation vials: Super	Packard	Frankfurt
Polyethylen		
Syringe	Beckton-Dickinson	Heidelberg

Chemicals

Chemicals	Supplier	Location
Acetic acid	Riedel de Haen	Seelze
Acrylamide mixture 37,5:1, 40%	Roth	Karlsruhe
Agarose SMG	AppliChem	Darmstadt
Ammonium hydroxide	Merck	Darmstadt
Ammonium peroxodisulfate	Merck	Darmstadt
1-Butanol	Riedel de Haen	Seelze
Bovine serum albumin (BSA)	Sigma	Taufkirchen
Calcium Chloride	KMF Laborchemie Handels GMBH	Lohmar
Chloroform	Riedel de Haen	Seelze
Complete Mini, EDTA-free Protease Inhibitor	Roche	Mannheim
Coomassie-blue R260	Serva	Heidelberg
DMSO	AppliChem	Darmstadt
DEAE-Sephadex A-25	Pharmacia LKB Biotechnology	Uppsala, Sweden
EDTA	Merck	Darmstadt
Enhancer	ICN	Meckenheim
Ethanol	Riedel de Haen	Seelze
Ethidium Bromide	AppliChem	Darmstadt
Foetal bovine serum	PAA Laboratories	Pasching, Österreich
Glycerol	Sigma	Taufkirchen
Glycine	Sigma	Taufkirchen

HEPES	Merck	Darmstadt
Hydrochloric acid, 37%	Riedel de Haen	Seelze
Lipopolisaccaride (LPS) from <i>E. coli</i> 0111:B4	Sigma	Taufkirchen
Methanol	Riedel de Haen	Seelze
Potassium chloride	Merck	Darmstadt
PD98059 (2-(2-Amino-3-methoxyphenyl)-4 <i>H</i> -1-benzopyran-4-one	Biotrend	Köln
Penicillin/Streptomycin	Biomol GmbH	Hamburg
Potassium dihydrogenphosphate	Merck	Darmstadt
Roti-Block	Roth	Karlsruhe
Roti-Load 1	Roth	Karlsruhe
Silica gel Lichropep RP18	Merck,	Darmstadt
Sodium acetate	Merck	Darmstadt
Sodium azide	Riedel de Haen	Seelze
Sodium chloride	Merck	Darmstadt
Sodium dodecylsulfate (SDS)	Sigma	Taufkirchen
Sodium desoxycholate	Roth	Karlsruhe
Sodium hydrogenphosphate	Merck	Darmstadt
Sodium hydroxide	Merck	Darmstadt
Sodium fluoride	Merck	Darmstadt
Sodium orthovanadate	Merck	Darmstadt
Sodium pyrophosphate	Merck	Darmstadt
N, N, N', N'-Tetramethylethylendiamin (TEMED)	Roth	Karlsruhe
Tricin, ultra pure	ICN	Meckenheim
Tris, ultra pure	ICN	Meckenheim
Triton X-100	Sigma	Taufkirchen
Tween-20	Sigma	Taufkirchen

Kits

Kit	Supplier	Location
CellTiter-Blue Cell Viability Assay	Promega	Mannheim
IL-1 α , IL-1 β , IL-6 ELISA Kit	Biosource	Solingen

PGE ₂ EIA Kit	Cayman Chemical, IBL-Hamburg	Hamburg
Western Blot Chemiluminescence Reagent Plus (ECL-Kit)	Roche	Mannheim

Antibodies, enzymes, proteins

Item	Supplier	Location
Cyclooxygenase-2 antibody	Santa Cruz Biotechnology	Heidelberg
goat anti-mouse IgG labelled with green fluorescent Alexa Fluor 488 dye	Invitrogen	Karlsruhe
MultiMark Multi-Colored Standard	Invitrogen	Karlsruhe
Phospho-JNK/SAPK IgG antibody	NEB	Frankfurt a. M.
JNK/SAPK IgG antibody	NEB	Frankfurt a. M.
Phospho-p38 MAPK IgG antibody	NEB	Frankfurt a. M.
p38 MAPK IgG antibody	Santa Cruz Biotechnology	Heidelberg
phosphor-p44/42 MAPK antibody	NEB	Frankfurt a. M.
p44/42 MAPK antibody	NEB	Frankfurt a. M.
Peroxidase-conjugated goat- anti-rabbit-IgG-antibody	NEB	Frankfurt a. M.
Peroxidase conjugated goat- anti-mouse-IgG-antibody	NEB	Frankfurt a. M.
Pertusis toxin (PTX)	Sigma	Taufkirchen
Protein-G-HRP conjugate	Bio-Rad	München
α -tubuline IgG antibody	Santa Cruz Biotechnology	Heidelberg
smooth muscle α -actin antibody	Dianova	Dörentrop, Germany
Recombinant rat IL-1 β , TNF- α	Biosource	Solingen
Trypsin/EDTA	Cambrex	Verviers, Belgium

Lipids

Lipid	Supplier	Location
Ceramide	Avanti Polar Lipids	Alabaster, USA
C8 Ceramide-1-phosphate	Biomol	Hamburg
C12 Ceramide-1-phosphate	Avanti Polar Lipids	Alabaster, USA
C18 Ceramide-1-phosphate	Sigma	Taufkirchen
Phosphatidic acid, didecanoyl sodium salt	Sigma	Taufkirchen
Sphingosin-1-phosphate (S1P)	Sigma	Taufkirchen

Radioactivity

Item	Supplier	Location
[1- ¹⁴ C] Arachidonic acid	Amersham Bioscience	Uppsala, Sweden
[γ - ³² P]-ATP	Amersham Life Science	Freiburg

Media

Medium	Supplier	Location
DMEM-F12	Invitrogen, Gibco	Karlsruhe
MEM	PAA Laboratories	Pasching, Austria
RPMI	Cambrex Bio Science	Verviers, Belgium

Solutions and buffers

Anode buffer (SDS-PAGE), pH 8.9	Tris-HCl	200 mM
APS	Ammonium peroxodisulfate	10% (w/v)
Catode buffer, SDS-PAGE, pH 8.25	SDS	0.1% (w/v)
	Tricine	100 mM
	Tris-HCl	100 mM
Coomassie-blue R260-Stock solution	Coomassie-blue R260	0.05% (w/v)
	Ethanol	25 %
Copper sulphate reagent	85% Phosphoric acid	50% (v/v)
	85% Phosphoric acid	8% (w/v)
	Copper sulfate	10% (w/v)
EDTA Solution, pH 8.0	EDTA (Dinatriumsalt)	500 mM

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3 x Gel buffer, pH 8.45	SDS	0.3% (w/v)	
	Tris-HCl	3 M	
Glycin-Strip buffer, pH 2.2	Glycin	0.2 M	
	SDS	0.1% (w/v)	
	Tween-20	1% (v/v)	
HBSS (Hank's balanced salt solution)	Perbio Science	Bonn Germany	
	phosphate buffer	0.1 M	
Homogenization buffer, pH 7.4 (buffer A)	glycerol	20%	
	mercaptoethanol	1 mM	
	EDTA	1 mM	
	sodium orthovanadate	1 mM	
	sodium fluoride	1 mM	
	leupeptin and aprotinin	10 µg/ml	
	PMSF(phenylmethylsulfonylfluoride)	1 mM	
	4-deoxypyridoxine	0.5 mM	
	Lysis buffer, pH 7.4	Complete Mini, EDTA-free	1 Tbl./10 ml
		Protease-Inhibitor	
		Desoxycholate, 5 % Stock solution	10% (v/v)
EDTA		2 mM	
β-Glycerophosphate		50 mM	
HEPES, pH 7.4		20 mM	
NaCl		100 mM	
Sodium flouride		10 mM	
Sodium pyrophosphate		10 mM	
orthovanadate		2 mM	
SDS 10% stock sol.		10%(v/v)	
Triton X-100, 10% stock sol.	10 % (v/v)		
PBS (Phosphate Buffered Saline)	KCl	3 mM	
	KH ₂ PO ₄	1,5 mM	
	NaCl	140 mM	
	Na ₂ HPO ₄	16 mM	
Reaction buffer (buffer B)	Tris buffer pH 7.4	30 mM	
	glycerol	5%	
	EDTA	0.25 mM	
	mercaptoethanol	1 mM	
	sodium orthovanadate	1 mM	

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	sodium fluoride	15 mM
	leupeptin , aprotinin	10 µg/ml
	4-deoxypyridoxine	0.5 mM
	PMSF	1 mM
Resolving gel, 10 %, 15 ml sol.	3 x Gel buffer	6.75 ml
	Acrylamide mix 37,5: 1, 40%	3.75 ml
	Glycerol	2.1 ml
	H ₂ O	2.25 ml
	APS 10%	15 µl
	TEMED	30 µl
Ringer solution	Baxter, Deerfield, III	USA
Stacking gel, 4 %, 7.5ml	3 x Gel buffer	3.415 ml
	Acrylamide mix 37.5: 1, 40%	750 µl
	H ₂ O	2.85 ml
	APS 10%	15 µl
	TEMED	70 µl
SDS-stock solution	SDS	10% (w/v)
S1P-stock solution	S1P, in Methanol	1.3 mM
50 x TAE, pH 8.0	EDTA	50 mM
	Tris-Acetate	2 M
TBS Blotto A	Non-fat dry milk	5%
	Tween-20	0.05%
TBST, pH 7.6	Sodium chloride	137 mM
	Tris-HCl	20 mM
	Tween-20	0.1% (v/v)
Transfer buffer, Western Blot, pH 8.5	Tris-Base	25 mM
	Glycin	200 mM
	Methanol	20% (v/v)
Triton X-100-stock solution	Triton X-100	10% (v/v)
Vanadate stock solution	Sodium orthovanadate, activated	200 mM

2.2. Methods

2.2.1. Animals

Male Sprague-Dawley rats (200-250 g) were obtained from Harlan Winkelmann (Borchen, Germany). Rats were subjected to surgical manipulation that involved the compression of the small intestine. These experiments were performed at the “Klinik und Poliklinik für Allgemein-, Viszeral-, -Thorax- und Gefäßchirurgie” of University of Bonn by Dr. Sven Wehner. Animal experiments followed institutional guidelines and were performed after approval of local ethics committees at University of Bonn. All animals were maintained on a 12-hour light/dark cycle with nutrition and tap water offered *ad libitum*.

2.2.1.1. Surgical manipulation model of rat small intestine

The small bowel of the animals was subjected to a nontraumatic intestinal manipulation according to standardized protocols [Kalff et al., 1998]. Rats (Stamm Wistar, SD, Lewis; 17 days old or younger) were anesthetized by inhalation anaesthesia, and a midline incision was made into the peritoneal cavity. The small bowel was eviscerated to the left onto a moist cotton gauze pad, and the entire small bowel was lightly manipulated with two moist cotton applicators. The operative procedures were performed under sterile conditions. Non-operated animals served as controls. In the time-course study, 3 groups of 5 animals were evaluated, each at a different time point (3, 6, 12, and 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.

2.2.1.2. *In vivo* induced endotoxemia

Endotoxemia was induced by bolus intraperitoneal injection of LPS (10 mg/kg). Rats were killed at various time points (3, 6, 12 and 24 h) after treatment for analysing the sphingolipid composition of the small intestinal tissue [Eskandari et al., 1997].

2.2.1.3. Small-bowel preparation

Animals were anesthetized at sacrifice and small-bowel specimens were harvested as described previously [Kalff et al., 1998]. The abdominal wound was reopened and the abdominal aorta cannulated and flushed with 3 ml of cold (4°C) Ringer solution. This flush was used to remove non-adherent and non-extravasated blood cells from the *muscularis*

vasculature. The entire small bowel was then removed and placed in cold pre-oxygenated Krebs-Ringer buffer (KRB). The small bowel was used for the selective isolation of the *muscularis* and *mucosa* layers. The entire small bowel was cut in 4- to 5-cm segments. Each segment was sequentially pinned down in a dissecting Sylgard dish containing chilled Hank's balanced salt solution (HBSS) to remove the mesenteric tissue before it was slipped onto a glass rod. A knitting needle with adequate size was inserted into each portion of intestine and carved along the mesenterium with a fine tweezers, cautiously not to damage the muscle layer. The *muscularis* was incised carefully along the mesentery and stripped of the *mucosa* by circumferentially peeling off the *muscularis* with a moist cotton applicator and placed in a glass filled up with chilled HBSS. The isolated layers were frozen in liquid nitrogen and stored at -80°C until tissue extraction. *Mucosa* and *muscularis* layers were used for lipid analysis.

2.2.2. Cell culture

2.2.2.1. Isolation of rat intestinal smooth muscle cells

Muscle strips were cut into very small pieces and transferred in a falcon tube filled up with HBSS. After they settled down, the HBSS was removed and a mixture of enzyme containing 0.6 mg/ml Collagenase Typ II, 2 U/ml Dispase II in 8 ml of HBSS was added. Strips were incubated at 37°C for 45 min in a shaking water bath at 120-140 rpm. After centrifugation for 5 min at 300 x g, the supernatant was discarded and the pellet was resuspended in 10 ml HBSS. Rat intestinal smooth muscle (RISM) cells dispersed from the *muscularis* layer were harvested by filtration through 500 µm Nitex mesh and centrifuged at 300 x g for 5min. Cells were resuspended and washed twice by centrifugation at 300 x g for 5 min. After resuspension in 5 ml of RPMI medium, 3 ml of Accuspin were added carefully for density gradient centrifugation, performed for 20 min, 800 x g, 4°C, and swing out 1/1. In the meantime the culturewares were coated with a thin film of serum and placed in the incubator at 37°C.

After gradient centrifugation, two phases and a pellet containing muscle cells were obtained. The pellet containing smooth muscle cells was transferred to a new 15 ml falcon tube. Cells were washed twice with 5 ml of RPMI by centrifugation at 300 x g, 5 min. The pellet was resuspended with 3 ml of Dulbecco's Modified Eagle Medium (DMEM-F12) medium. Cells were counted using trypan blue solution in 1:1 ratio with cell suspension. Cells were incubated in a 10% CO₂ atmosphere at 37°C.

Culture medium (DMEM/F12 + 10% fetal calf serum + 1% Pen/Strep) was changed every three days until the cells reached confluence. The cells were used in an early passage because after 5-6 passages they differentiate to fibroblasts or myofibroblasts.

2.2.2.2. Splitting of smooth muscle cells

When a postconfluent density was reached, the cell culture was split 1:3 –1:5 in 75 cm² flasks by removing the culture medium, washing the cell monolayer with warmed Phosphate Buffer Saline (PBS) and pipetting 2-3 ml (0.05% / 0.02%) of Trypsin/EDTA for 2-3 min. During that time, the flask was placed in the incubator and the cells were examined microscopically to ensure that all of them were detached and floating. The side of the flask was gently tapped to release any remaining attached cells. The trypsin was inactivated with 5 ml of culture medium and the cell suspension was pelleted for 5 min at 300 x g. The supernatant was removed, the cells resuspended in culture medium and added to flasks (75 cm²) containing 15 ml of medium.

2.2.2.3. Storage of smooth muscle cells

After splitting the cells (trypsinization and centrifugation), the pellet was resuspended in 2 ml of foetal calf serum (FCS) and 2 ml of cell culture freezing medium (serum free medium DMEM-F12 with 10% DMSO). Cell suspension was aliquoted into appropriate cryogenic storage vials (2 ml/vial) and placed at -80°C for 24 h and then in liquid nitrogen (-196°C).

2.2.2.4. Treatment of smooth muscle cells with sphingolipids and other compounds

Confluent cells from 75 cm² flasks were harvested as described above and placed in 8 cm² or 21 cm² dishes. The dilution of the cells was adjusted to achieve approximately 80% confluency at the time of treatment. Experiments were performed in Modified Eagle Medium (MEM) supplemented with 0.3% FCS and 1% Pen/Strep. At the day of treatment, the culture medium was removed, the cell monolayer was washed twice with the new medium and the cells were incubated for different time periods with medium containing methanol (as control) or different sphingolipids. The final concentration of shingolipids in medium was 1, 5 or 10 µM. S1P was added to the medium as complex with bovine serum albumin (BSA) (4 mg/ml). The complex was formed by incubating the BSA-medium with S1P for 30 min at 37°C. In some experiments the cells were preincubated for 30 min with medium containing inhibitors

of the signal transduction cascade components (ERK, p38 MAPK, receptor coupled $G_{i/o}$ -protein) prior to being treated with sphingolipids.

At different time intervals after treatment, the cells were harvested with a cell scraper in 500 μ l of PBS (4°C) after removing the medium and washing twice with cold PBS. The cell suspension was pelleted at 2000 x g for 10 min, 4 °C and cell pellets were stored at -37 °C until their subsequent use.

2.2.2.5. Immunocytochemistry

For immunofluorescence experiments, cells were plated on glass coverslips for up to 72 h. When cells were 50-70% confluent, the culture medium was removed, and the cells were thoroughly washed with pre-cooled PBS. The pre-washed cells were fixed with cold methanol for 10 min at -20°C. Fixed cells were washed three times with PBS to remove any solvent traces and the reaction was blocked by incubation in PBS containing 3% BSA for 1 h at room temperature. Cells were washed one time with 0.3% PBS/ BSA and then incubated overnight at 4°C with a smooth muscle α -actin primary antibody (1:100). Coverslips were washed three times with PBS and then incubated for 1 h at room temperature with secondary antibody, goat anti-mouse IgG labelled with green fluorescent Alexa Fluor 488 dye. Immunoreactive protein was visualized by fluorescence microscopy.

2.2.2.6. Application of mechanical stretch to smooth muscle cells

RISM cells were seeded into 6-well type 1 collagen-coated, silicone elastomer-base culture plates. The cells grown to confluence on the flexible surface were subjected to cycles of stretch and relaxation using a computer-driven, vacuum-operated, stress-providing instrument (Flexercell Strain Unit FX-4000 Tension plus; Flexcell Interational). The vacuum induced 20% elongation in the diameter of the flexible surface with sinus shapes. The cells were exposed to stretch for 2 x 30 min (0.5 Hz) 5 min break in between. Mechanical stretch was performed in a humidified incubator with 5% CO₂ and 37°C. Control cells were grown in nondeformable plates in parallel. Cells were examined by phase-contrast microscopy to verify cell attachment.

2.2.3. Lipid analysis by thin layer chromatography

2.2.3.1. Extraction of lipids from tissues

Tissue samples collected after gentle manipulation of the small bowel were provided by Prof. Jörg Kalff from Department of Surgery, University of Bonn and stored at -80°C . Before starting the procedure, an appropriate amount of intestinal tissue was weighted (about 50 mg wet weight from *mucosa* layer and 25 mg wet weight from *muscularis* layer, respectively). The samples were homogenized with 600 μl of water (bi-distilled water) using a Mixer-mill. Homogenates were transferred to glass tubes and lipids were extracted with 2 ml of methanol and 1 ml chloroform for 48 h at 40°C . After extraction, tissue debris were spinned down by centrifugation at 2000 rpm for 10 min at room temperature and the upper phase was filtered through glass Pasteur pipettes filled with cotton wadding. The pellet was extracted again with 700 μl water and 3 ml of chloroform/methanol (1:2; v/v) by strong vortexing for 30 sec. The upper phases were pooled and the solvent was evaporated under a stream of nitrogen at 37°C .

2.2.3.2. Extraction of lipids from smooth muscle cells

The cell pellet was homogenized in 400 μl of water and at this step aliquots were taken for protein determination. Total lipids were extracted with 5.85 ml of chloroform/methanol (2:1; v/v) for 24 h at 48°C . After extraction, denaturated particles were removed by passing the samples through glass Pasteur pipettes filled with cotton wadding, used as filtration columns. Prior to application of the lipid extract, the wadding filter was rinsed with 1 ml of extraction solvent chloroform/methanol/water (10:5:1 v/v/v). The filter sample was collected in a new screw-capped Pyrex tube with Teflon inlay placed under the pipette tip. Finally, the wadding filter was rinsed with 2 ml of extraction solvent that was collected in the same tube. The solvent was evaporated under a stream of nitrogen at 37°C [van Echten-Deckert, 2000].

2.2.3.3. Removal of lipid contaminants

Phospholipids, the major lipid components of the biological membranes comigrate with different sphingolipid species during their separation by thin layer chromatography (TLC). Therefore they were removed from the cellular lipid extracts by mild alkaline methanolysis (saponification). Extracts were dissolved in 2.5 ml of methanol and sonicated for 5 min in a sonifier. Then, 62.5 μl of NaOH (4 M stock solution in water) were added to a final concentration of 100 μM . After shaking for 2 h at 37°C , samples were neutralized by addition of 10 μl of concentrated acetic acid. Finally, the solvent was evaporated as described above.

2.2.3.4. Reversed-phase chromatography

Small hydrophilic molecules such as salts, amino acids, sugar, small peptides are extracted along with sphingolipids, as well as salts formed by addition of NaOH, interfere with lipid behaviour during separation by TLC. The polar nonlipid contaminants were removed by reversed-phase chromatography [van Echten-Deckert, 2000].

2.2.3.4.1. Preparation of gel

Silica gel RP18 (particle size 40-63 μm) was suspended in chloroform-methanol (2:1, v/v) and cautiously shaken for 30 min. The silica gel was allowed to settle and after removing the supernatant, the silica gel was resuspended in methanol (1:1, v/v), shaken carefully for 30 min, and the supernatant was poured off again. This step was repeated 3-4 times with fresh methanol. The suspended gel was stored in methanol at 4 °C.

2.2.3.4.2. Chromatography

A small piece of fibre wadding was introduced into glass Pasteur pipettes and 2 ml of the silica gel RP18 suspension was added. The columns were rinsed two times with 1 ml of chloroform/methanol/0.1 M potassium chloride (6:96:94, v/v/v). The samples (lipid extracts) were dissolved in 1 ml of methanol and sonicated for 5 min. Then 1 ml of ammonium acetate (300 mM in water) was added to each sample.

The samples were applied onto the columns. The empty sample tubes were rinsed two times with 0.5 ml of ammonium acetate (200 mM in methanol/water, 1:1, v/v) and applied to the columns. The bulk of polar contaminants remained in the flow-through fraction. The columns were washed with 6 ml of water (doubly distilled quality) to elute all polar contaminants. New tubes were placed under each column for collecting the lipid fraction. The lipids were eluted with 1 ml of methanol and with 8 ml of chloroform-methanol (1:1, v/v). The samples were dried by evaporating the solvent under a stream of nitrogen.

2.2.3.5. Anionic-exchange chromatography

Anionic glycosphingolipids, such as gangliosides and sulfatides, can be separated from neutral glycosphingolipids, sphingomyelin, and ceramide by anionic-exchange chromatography. In this way, comigration of anionic and neutral glycosphingolipids on the same TLC can be avoided.

2.2.3.5.1. Preparation of resin

DEAE-Sephadex A-25 was used in our study. For stability reasons the resin was delivered in its chloride form and had to be converted to the acetate form.

DEAE-Sephadex was let to swell overnight in distilled water (10 g in 150 ml). The supernatant was discarded by filtration using a Chinese funnel and filter paper. The resin was washed again with distilled water and then with 1 M sodium acetate in water until the supernatant was devoided of chloride anions. This was tested by acidification of the discarded supernatant with silver nitrate. Conversion of DEAE-Sephadex to the acetate form was completed when no silver chloride precipitate was formed in the supernatant. The DEAE-Sephadex in its acetate form was stored in methanol (1:1) at 4°C.

2.2.3.5.2. Chromatography

Small pieces of cotton wadding were introduced into glass Pasteur pipettes and 2 ml of DEAE-Sephadex suspension were added. The columns were then washed with 1 ml of methanol and 3 times with 1 ml of chloroform/methanol/water (3:7:1, v/v/v). A new tube was placed under each column for collection of the neutral lipids. The samples were dissolved in 1 ml of solvent chloroform/methanol/water (3:7:1, v/v/v) and sonicated for 1 min in an ultrasonifier.

The samples were applied to the columns and the empty sample tubes were rinsed two times with chloroform/methanol water (3:7:1, v/v/v) and applied to the columns. Neutral lipids were eluted with 3 ml of chloroform/methanol/water (3:7:1, v/v/v) and after evaporating of the solvent this lipid fraction was ready for TLC. New tubes were placed under each column for collection of the anionic lipid fraction. Anionic lipids were eluted with 8 ml of chloroform/methanol/0.8 M ammonium acetate (3:7:1, v/v/v). The anionic lipid fractions were dried and salts were removed by reversed-phase chromatography as described above.

2.2.3.6. Thin layer chromatography

Glass-backed silica gel 60 precoated TLC plates were used for sphingolipid thin layer chromatography (TLC). Samples (maximal volume 50 µl) were applied as 0.5-to 1-cm bands on the TLC plate using small glass capillaries. Solvent systems (mixtures of chloroform/methanol/water or aqueous salts) used for development of chromatograms were freshly mixed and added to the tank allowing the formation of the vapour-liquid equilibrium for at least 60 min.

For TLC separation of most sphingolipids we used the following solvent system: chloroform/methanol/0.22% aqueous CaCl₂, (60:35:8, v/v/v). For a good resolution of ceramide, the TLC plate was run twice consecutively in chloroform/methanol/acetic acid (190:9:1, v/v/v). Neutral sphingolipids (sphingomyelin, lactosylceramide, ceramide, cholesterol) were resolved using the solvent system: chloroform/methanol/2 M aqueous ammonia (65:25:4, v/v/v).

2.2.3.7. Visualization and quantitative determination of sphingolipids on TLC

2.2.3.7.1. Autoradiography

Cellular sphingolipids biosynthetically labelled with L-[3-¹⁴C]serine were detected and evaluated on TLC plates with the bioimaging analyser Fujix Bas 1000 using the software TINA 2.09 (Raytest, Straubenhardt, Germany).

2.2.3.7.2. Staining

For visualization of non-radioactive sphingolipids we used cupric sulphate in aqueous phosphoric acid. The TLC plates were sprayed heavily until they appeared wet. Then the plates were heated in an oven at 180°C for about 15 min. Lipid bands obtained by TLC were evaluated by photodensitometry (Shimadzu, Kyoto, Japan).

2.2.4. Lipid analysis by mass spectrometry

Sphingolipids from rat small bowel tissue (*mucosa* and *muscularis* layers) were analysed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) in laboratory of Professor Alfred H. Merrill Jr. at Georgia Institute of Technology (Atlanta, USA) according to a standard protocol [Sullards and Merrill, 2001].

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

2.2.4.1. Extraction of samples

Prior to extraction, tissues were weighed, homogenized in ice cold PBS, transferred into 13 x 100 mm borosilicate screw cap test tubes with Teflon caps and frozen-dried/lyophilized in triplicates. The aqueous volume at this stage was 0.1 to 0.2 ml. After adding 0.5 ml of methanol and internal standards (in 10 μ l), the lipids were dispersed using a bath type sonicator at room temperature for 30 sec, 0.25 ml of chloroform were added, and the capped tubes were incubated at 48°C overnight in a heating block. After cooling, 75 μ l of 1 M KOH in methanol were added, mixed briefly by sonication, incubated in a heating block for 2 h at 37°C, and clarified by centrifugation. For analysis of sphingoid bases and related compounds, 0.4 ml was removed and transferred to a new test tube and the solvent was evaporated under vacuum at room temperature (in a Savant Speedvac). The lipids were redissolved afterwards by sonication of the mobile phase for reversed-phase chromatography, centrifuged to clarify and transferred to autoinjector vials. The remainder of the extract was neutralized with 3 μ l of glacial acetic acid, and 1 ml of chloroform and 2 ml of water were added. The samples were mixed and then centrifuged, the lower layer was collected with care to recover the interface, transferred to a new test tube, and the solvent was removed under vacuum at room temperature. In the end, the lipids were redissolved in the mobile phase for normal phase chromatography with sonication, centrifuged to clarify and transferred to autoinjector vials.

2.2.4.2. High Performance Liquid Chromatography Tandem Mass Spectrometry

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. x 5 cm Discovery C18 column and a flow rate of 1 ml/min. Mobile phase A consisted of methanol/water/formic acid (58:41:1, v/v/v); mobile phase B consisted of methanol/formic acid (99:1, v/v); 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 μ l 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 and then the column was re-equilibrated at initial conditions for 0.5 min.

Complex sphingolipids (ceramide, sphingomyelin) were separated by normal phase chromatography using a Supelco 2.1 mm i.d. x 5 cm LC-NH₂ column and a flow rate of 1.0 ml/min. Mobile phase A consisted of acetonitrile/methanol/acetic acid (97:2:1, v/v/v); mobile phase B consisted methanol/water/*n*-butanol/acetic acid (64:15:20:1, v/v/v/v); 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 was 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 N₂ was used as the nebulizing gas at a flow rate of 6 l/min. The ion spray 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 N₂ drying gas temperature was set to 500 °C. N₂ 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 the variants with a 4-hydroxysphinganine backbone [Merrill et al., 2005]. 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 that are usually within + 10%. Quantitative analysis was based on calibration curves constructed by plotting the peak area ratios of analyte to the respective internal standard against concentration using a linear regression model. The target analyte peak area ratios from the samples were normalized to the respective internal standard and compared with the calibration curves.

2.2.5. Sphingosine kinase activity assay

For sphingosine kinase assay, RISM cells were washed and scraped in cold PBS. After centrifugation, the cell pellet was homogenized in 50 µl of phosphate buffer, pH 7.4 (buffer A) and cells were disrupted using a mixer-mill. For subsequent assays, the cell pellets were stored at -80°C without loss in sphingosine kinase activity.

For studies of sphingosine kinase activity, 70-100 µg proteins were used, with or without sphingosine (50 µM) delivered as sphingosine-BSA complex. The reaction mixture contained Tris buffer, pH 7.4 (buffer B) in a final volume of 200 µl. Reactions were started by addition of 10 µl of ATP in MgCl₂ (100 mM), [γ ³²P] ATP (1-2 µCi, 20 mM), and incubated for 30min at 37°C in a shaking bath. Reactions were stopped by adding 20 µl of 1 N HCl followed by

0.8 ml of chloroform/methanol/HCl (100:200:1, v/v/v). After vigorous vortexing, 240 µl of chloroform and 240 µl of 2N KCl were added and the phases were separated by centrifugation. The labelled lipids in the organic phase were resolved by TLC on silica gel G60 with 1-butanol/methanol/acetic acid/water (80:20:10:20, v/v/v/v) and visualized by autoradiography. The radioactive spots corresponding to authentic S1P were identified using S1P as standard.

2.2.6. Quantification of arachidonic acid release

Cells were labelled overnight with 0.2 µCi /ml of [¹⁴C] arachidonic acid in culture medium. Then medium was removed, cells were rinsed three times with culture medium and then incubated in 1 ml of medium containing the desired additives. After incubation for the indicated times, the medium was removed and an aliquot was submitted to scintillation counting. Results were normalized to cell protein. In addition, media were subjected to reversed-phase chromatography as described above (see section 2.2.3.4). The samples were applied on Silicagel-60 TLC-plates developed with n-hexane/diethyl ether/glacial acetic acid (70:30:1, v/v/v). Arachidonic acid was visualized by autoradiography using the bio-imaging analyzer Fujix bas 1000 software, Tina 2.09, (Raytest, Straubenhardt, Germany).

2.2.7. Immunoblotting

2.2.7.1. Preparation of protein samples

The cell pellets were resuspended in 100 µl of lysis buffer (4°C) (see section 2.1), incubated for 10 min on ice and shortly sonicated for 20 sec at 60 W. Cell debris were spinned down by centrifugation for 10 min, at 10000 x g, 4°C. The cell lysates were transferred to new eppendorf tubes and the concentration of protein was determined with Bradford method as described in section 2.2.7.2. The cell lysates were stored at -80°C until their subsequent use. For Sodium dodecylsulfate-Polyacrylamide gel electrophoresis (SDS-PAGE), a corresponding amount of cell lysate (as calculated after protein determination) was treated with a reducing SDS-containing buffer (RotiLoad 1) and heated up for 10 min at 95°C in a thermomixer. To separate the proteins, 10-20 µg of sample were applied to SDS-PAGE.

2.2.7.2. Quantitative measurement of protein concentrations

The protein concentration was determined with the Bradford method in a microtiter plate. This method is based on the blue stain of Coomassie blue in acidic solution upon reaction

with protein. A standard curve for BSA was calculated from the absorptions obtained from 10 standard solutions ranging from 0 to 250 $\mu\text{g/ml}$. Always, 20 μl of standard or protein sample at varying dilutions were added to 200 μl of Bradford reagent (Coomassie-Blue R250) diluted 1:5 in Millipore water. The microtiter plate was incubated for 10 min in the absence of light and the absorption measurements (595 nm) and calculation of sample concentrations were carried out photometrically on a Multiskan Ascent readout instrument.

2.2.7.3. SDS-PAGE

Proteins were separated on a 10% running gel and a 4% stacking gel according to Schagger-Jagow [Schagger and von Jagow, 1987]. Electrophoresis was performed in 8 x 9 cm vertical gels of 1 mm thickness in a Minigel apparatus. Gels were run at 60-120 V and in cool conditions to avoid the “smiling” effect of protein bands due to overheating.

2.2.7.4. Western Blotting

After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane by wet blotting. Before blotting, the nitrocellulose membrane was soaked in transfer buffer. The membrane was placed on the gel and covered with filter paper and sponge pads, both soaked in transfer buffer. The assembly was enclosed in the blotting apparatus filled with cooled transfer buffer. Blotting was performed for 2 h at 250 mA. After the protein transfer, the membrane was saturated with 5% non-fat dry milk in Tris-Buffered Saline Tween-20 (TBST) or with 1x RotiBlock for 1 h at room temperature. For protein identification, the membrane was incubated in a solution containing appropriate primary antibody, overnight at 4°C, on a rotator. After incubation, the membrane was washed 3 times for 10 min each, with TBST and incubated with the appropriate secondary antibody (anti-mouse-HRP 1:10000, anti-rabbit-HRP 1:10000, protein G-HRP-conjugate 1:10000) in TBST for 2 h at room temperature. After further washing steps in TBST, the membrane was incubated for 1 min with 1.5 ml of enhanced chemiluminescence reagent. The chemiluminescence of the secondary antibody was detected by a photographic film (10 sec to 3 min depending on the chemiluminescence intensity). The same membrane was used to identify proteins that are constitutively expressed in the cells in order to confirm equal amounts of protein loaded for each sample. For that, the blots were stripped twice for 15 min with Glycin-stripping buffer to remove the bound antibodies. After washing 2 times, 10 min each, with TBST and after blocking, the membrane was reprobated with an antibody specifically recognizing total (phosphorylated and

unphosphorylated) MAPKs or α -tubulin for membranes first treated with cyclooxygenase-2 antibody.

Primary antibody	Donor	Dilution
Anti-cyclooxygenase-2	goat	1:400 in TBSBlottoA
Anti-phospho-p38 MAPK	Rabbit	1:1000 in TBST
Anti-p38 MAPK	Rabbit	1:1000 in TBST
Anti-phospho-p44/42 ERK	Rabbit	1:1000 in TBST
Anti-p44/42 ERK	Rabbit	1:1000 in TBST
Anti-phospho-JNK/SAPK	Rabbit	1:1000 in TBST
Anti-JNK/SAPK	Rabbit	1:1000 in TBST
Anti- α - tubulin	mouse	1:5000 in TBST

2.2.8. Enzyme Linked-Immuno-Sorbent Assay

2.2.8.1. Prostaglandin E₂ assay

Cells were seeded in 8 cm² dishes and treated with sphingosine-1-phosphate or ceramide-1-phosphate as described in section 2.2.2.4. At different time intervals, media were collected and centrifuged at 1000 g x 5 min to remove detached cells and stored at -80 °C for subsequent PGE₂ determination. A 1:20 dilution ratio was optimal for analysis. Secreted PGE₂ was assayed using the Prostaglandin E₂ monoclonal enzyme immunoassay (EIA) kit. This assay is based on the competition between PGE₂ presented in the media and a PGE₂-acetylcholinesterase (AChE) conjugate (PGE₂ Tracer) for a limited amount of PGE₂ monoclonal antibody. The antibody-PGE₂ conjugate binds to a goat anti-mouse antibody previously attached to the wells (Fig. 7). The plate is washed to remove any unbound reagent and then Ellman's reagent (which contains the substrate to AChE) is added to the wells. The product of this enzymatic reaction has a yellow colour and absorbs strongly at 412 nm. The concentration of PGE₂ present in a sample is inversely proportional to the yellow colour produced. The concentration of PGE₂ was calculated using a standard curve prepared with known concentrations of PGE₂. Samples were assayed in triplicate for each condition.

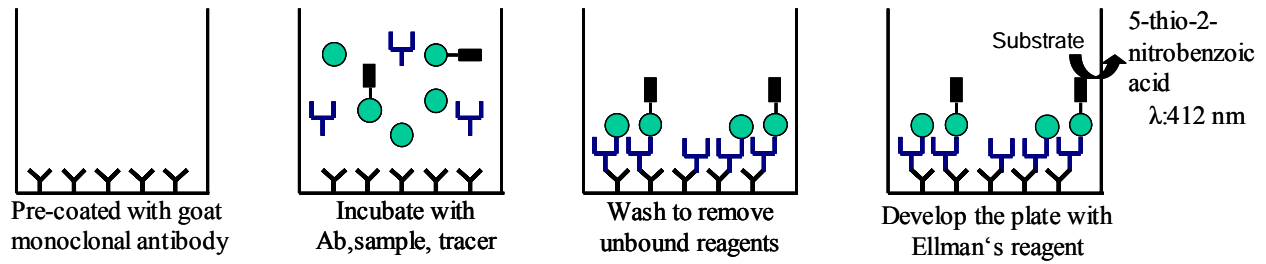


Fig. 7 Scheme of the PGE₂ enzyme immunoassay. Y- goat monoclonal anti-mouse IgG; Y-PGE₂ monoclonal antibody; ●■ -acetylcholinesterase linked to PGE₂ (Tracer); ●-PGE₂

2.2.8.2. Cytokine assays

Cytokines released in cell culture media (IL-1 β , IL-6) were measured using the respective ELISA kits. After treatment with S1P, TNF- α or LPS, cultured media were collected and centrifuged to remove detached cells and stored at -80°C for subsequent cytokine determination. ELISA for rat IL-1 β , IL-6 was performed according to the manufacture's instructions. These assays utilize an immobilized rat IL-1 β or IL-6 antibody, a biotinylated antibody specific for rat IL-1 β or IL-6, horseradish peroxidase-conjugated streptavidin and tetramethylbenzidine (TMB) as the peroxidase substrate. The optical density was determined using a Multiskan microplate reader set at 450 nm (Fig. 8) and the concentration of the respective cytokine was calculated using a standard curve prepared with known concentrations of recombinant rat IL-1 β or IL-6. Samples were assayed in triplicate for each condition.

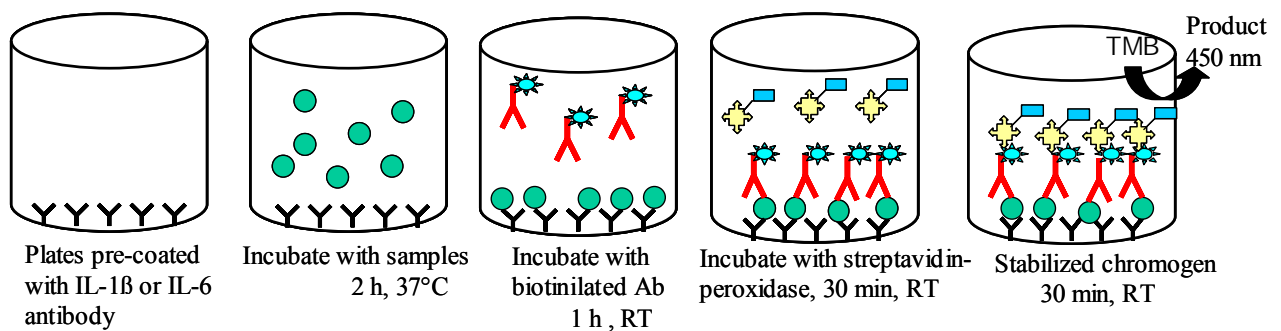


Fig. 8 Scheme of the cytokines enzyme immunoassay. Y-IL-1 β , IL-6-antibody; ●-IL-1 β or IL-6; Y★ biotin-labelled anti-IL-1 or IL-6; +■-streptavidin-peroxidase (HRP)

2.2.9. Statistics

Data were compiled as mean \pm standard deviation of the mean. Statistical analysis was performed using GraphPad Prism 3.03 software (GraphPad Software Inc. California, USA) with a significant level of *p<0.05. **p<0.01

3. Results

3.1. Standardized “in vivo” animal model of small intestinal manipulation

It has been previously shown that surgical manipulation of the rat small intestine elicits an inflammatory response within the *tunica muscularis externa* accompanied by suppression of smooth muscle layer contractility and hence a decreased intestinal transit. For those studies, a standardized surgical rodent model of small intestinal manipulation was established [Kalff et al., 1998]. Rats were subjected to progressive degrees of gentle surgical manipulation that involved compression of the small intestine to mimic normal abdominal operative procedures. In rats the small intestine is said to serve as a good model for postoperative ileus [Luckey et al., 2003]. To investigate the potential role of sphingolipids in postsurgical ileus, we used this animal model. As a result of intestinal manipulation, functional alterations of rat small intestine might be reflected by changes in the levels of sphingolipids. Therefore, we analysed sphingolipid composition of manipulated rat small intestine (*mucosa* and *muscularis*, respectively) at different time intervals: 0 h, 3 h, 6 h, 12 h and 24 h postsurgically. Recent studies on inflammation progressively revealed a key role of bioactive sphingolipids in inflammatory responses [Pettus et al., 2004b].

3.1.1. The content of sphingolipids in rat small intestine samples at different time intervals after surgical manipulation

3.1.1.1. Analysis of sphingolipids by thin layer chromatography

To determine the content of sphingolipids in rat small intestine before and after non-traumatic intestinal manipulation, *mucosa* and *muscularis* layers (50 mg and 25 mg wet weight, respectively) were collected at different time intervals (0 h, 3 h, 6 h, 12 h and 24 h) following surgery and subjected to lipid analysis. First, lipids were extracted with chloroform/methanol and analysed by thin layer chromatography (TLC) as described in section 2.2.3. Attention was initially focused on the content of ceramide, the primary sphingolipid found to play a role in inflammatory processes. Ceramide direct metabolic derivative, sphingomyelin (SM) was studied in parallel. To statistically analyse the content of sphingolipids in rat small intestine after surgical manipulation, three groups of samples were prepared and processed in independent experiments. For each sample, sphingolipid content was expressed as the percentage of total lipids extracted. Ceramide was detected in both rat intestinal *mucosa* and

muscularis, amounted about 4% of total lipids. The TLC data showed no significant change in the ceramide content in both layers and over the entire time range tested, when compared to non-manipulated controls (Fig. 9 A, B, C). As illustrated in Fig. 9 D, the content of its metabolic product, SM was found to slightly increase in *muscularis* versus *mucosa* (about 7% and 5%, respectively), with not statistically differences at any time interval postsurgery compared to control samples in both layers (Fig. 9 D). These studies provide evidence that intestinal manipulation do not induce changes in ceramide or SM composition of rat small intestine.

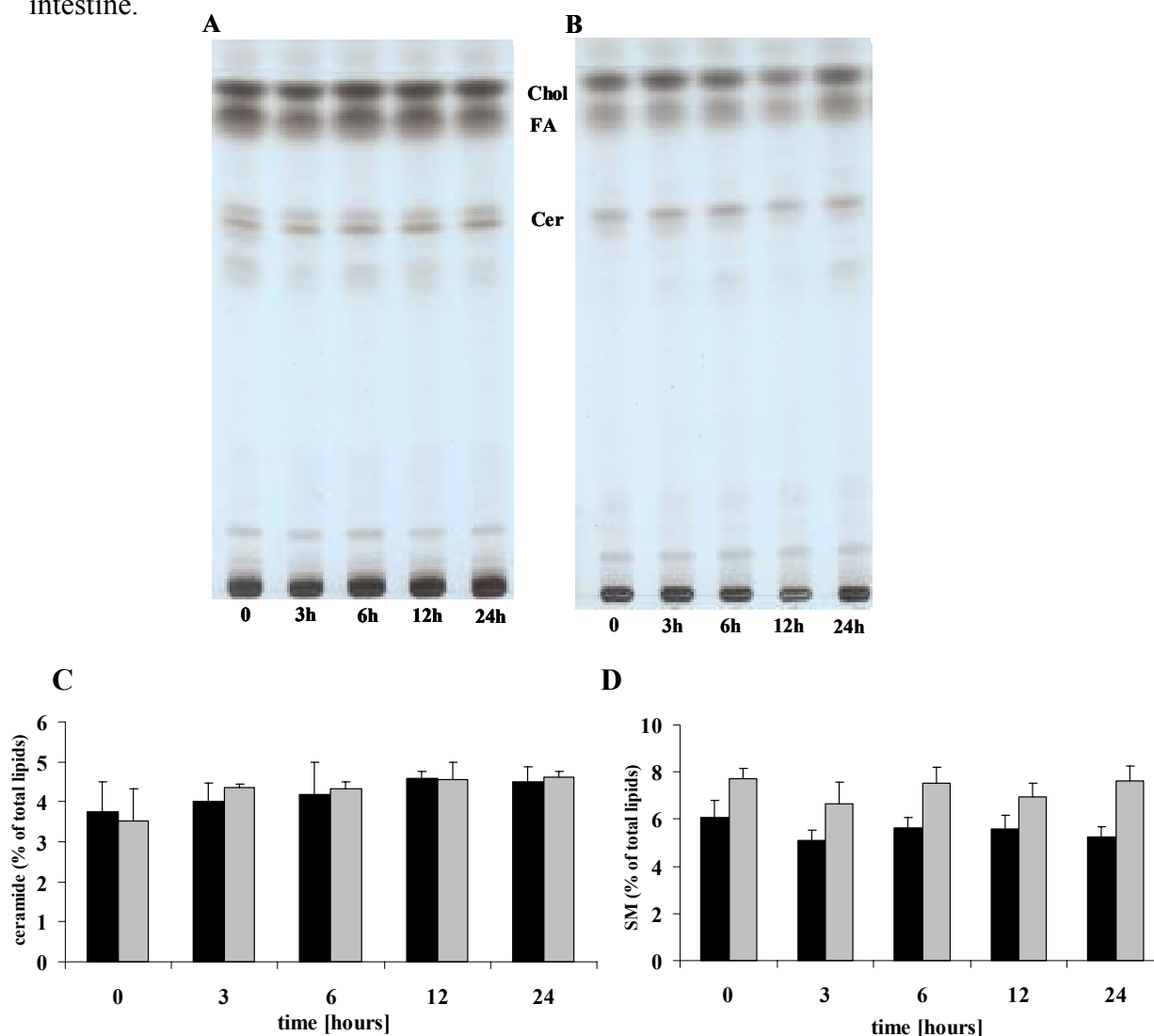


Fig. 9 Effect of surgical manipulation on ceramide level in intestinal *mucosa* and *muscularis*. The intestinal *mucosa* (A) and *muscularis* (B) were dissected as described in section 2.2.1.2, at indicated time intervals after manipulation. The specimens were homogenized in 600 μ l water and lipids were extracted with chloroform/methanol (1:2; v/v). After mild alkaline hydrolysis to remove glycerophospholipids, the lipid extracts were desalted and subjected to thin layer chromatography as described in sections 2.2.3.4, 2.2.3.6. In some experiments lipid extracts were subjected to anionic exchange chromatography. The TLC plate was run twice consecutively in chloroform/methanol/acetic acid (190:9:1; v/v/v). For detection of lipids TLC plate was sprayed with 8% phosphoric acid/10% cupric sulphate and developed for 10 min at 185°C. The mobility of standard lipids is indicated. C, D) Ceramide, SM (black columns, *mucosa*; grey columns, *muscularis*) were quantified by photodensitometry. Data are averages of three independent experiments. Cer, ceramide; FA, fatty acid; Chol, Cholesterol; SM, sphingomyelin.

3.1.1.2. Analysis of sphingolipids by mass spectrometry

High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) is currently the only analytical procedure with appropriate structural specificity, sensitivity and quantitative precision used for simultaneously analysis of many different sphingolipids. The method can detect changes in sphingolipid levels as low as 1pmol or even less [Merrill et al., 2005]. Note that these measurements were performed by Prof. A. Merrill at Georgia Institute of Technology, Atlanta. To measure the content of sphingolipids in rat small intestine before and after manipulation (Fig. 10 A), *mucosa* and *muscularis* tissue samples, collected at different time intervals postsurgery (0 h, 3 h, 6 h, 12 h, and 24 h) were subjected to HPLC-MS/MS. The results showed that at the indicated time intervals after manipulation and also at time zero, the amount (mol/mg wet weight) of all lipids analysed was higher in the *muscularis* layer than in *mucosa* (not shown). The most abundant sphingolipid was sphingomyelin with about 500 and 250 pmol/mg wet weight, followed by ceramide with almost 350 and 150 pmol/mg wet weight in *muscularis* and *mucosa*, respectively. No significant change in the level of these two sphingolipids was detected at any time interval postsurgically in comparison with controls, non-manipulated rat intestine samples, in both, *muscularis* (Fig. 10 B, C) and *mucosa* (not shown) thus confirming data obtained by TLC. HPLC-MS/MS also enabled us to accurately determine the levels of sphingoid bases and their phosphorylated counterparts. The amount of sphingosine was by one order of magnitude below the mass of ceramide, about 35 and 10 pmol/mg wet weight in *muscularis* and *mucosa*, respectively. Its content was statistically not different from controls at any post-traumatic interval tested in *muscularis* (Fig. 10 D) and *mucosa*, respectively. In both layers, the content of ceramide-1-phosphate (C1P) and of sphingosine-1-phosphate (S1P) clustered within a picomolar range, no significant changes of any of these two sphingolipid-phosphates in the *mucosa* layer after intestinal manipulation being observed (data not shown). In contrast to the *mucosa* layer, the intestinal *muscularis* exhibited a time dependent increase in the content of C1P and S1P (Fig.10 E, F). As illustrated in Fig. 10 E, F the time courses of expression of the two sphingolipid-phosphates were quite similar. Both peaked at 6 h after intestinal manipulation reaching a more than 3-fold higher level at this time point when compared to non-manipulated rat intestinal samples ($p < 0.01$, $n=3$). After 6 h, the level of C1P decreased slowly but continuously towards control values, whereas that of S1P remained elevated to some extent at longer time intervals after surgery. Notably, the amount of both sphingolipids appears to be subjected to a long-time up-regulation post-surgery.

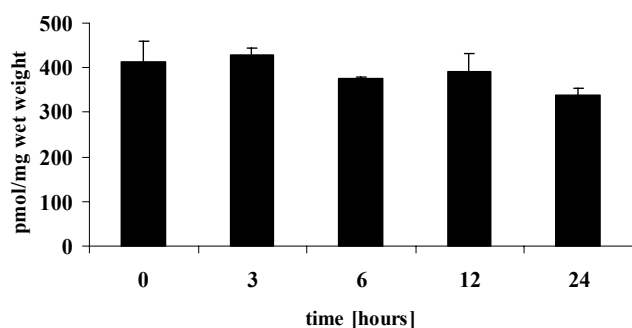
Hence, of the investigated sphingolipids only the content of C1P and S1P was increased, suggesting a possible role during the post-traumatic impairment of intestinal *muscularis* function. Moreover, these findings might indicate for the first time a direct involvement of these two bioactive lipids in the inflammatory responses within the intestinal *muscularis*, which has been suggested to play a major role in the pathogenesis of postoperative ileus [Kalff et al., 1998; Kalff et al., 1999b]. These results prompted us to focus our attention mainly on studies concerning *muscularis* layer rather than *mucosa*.

A



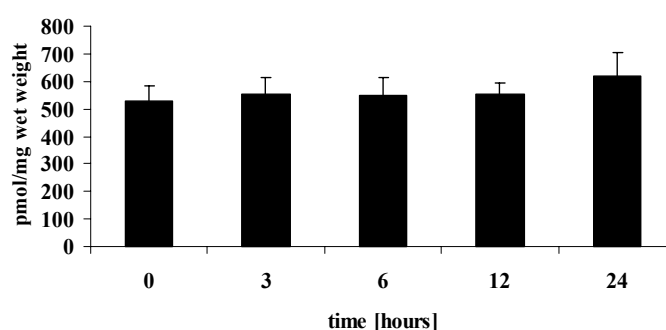
B

Ceramide (ME-IM)



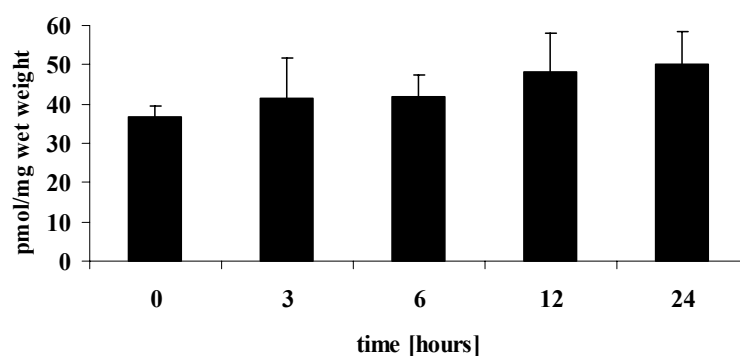
C

Sphingomyelin (ME-IM)



D

Sphingosine (ME-IM)



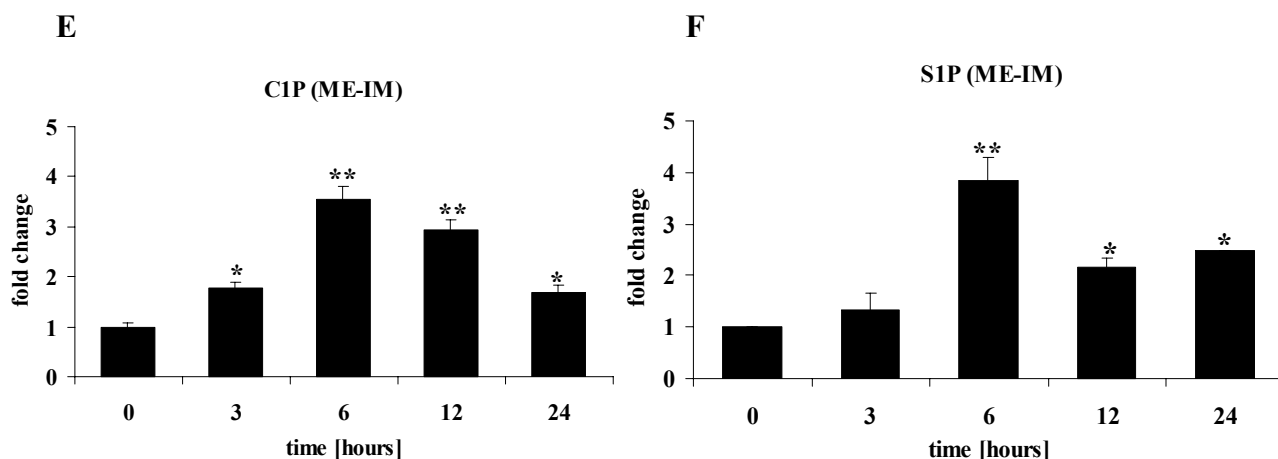


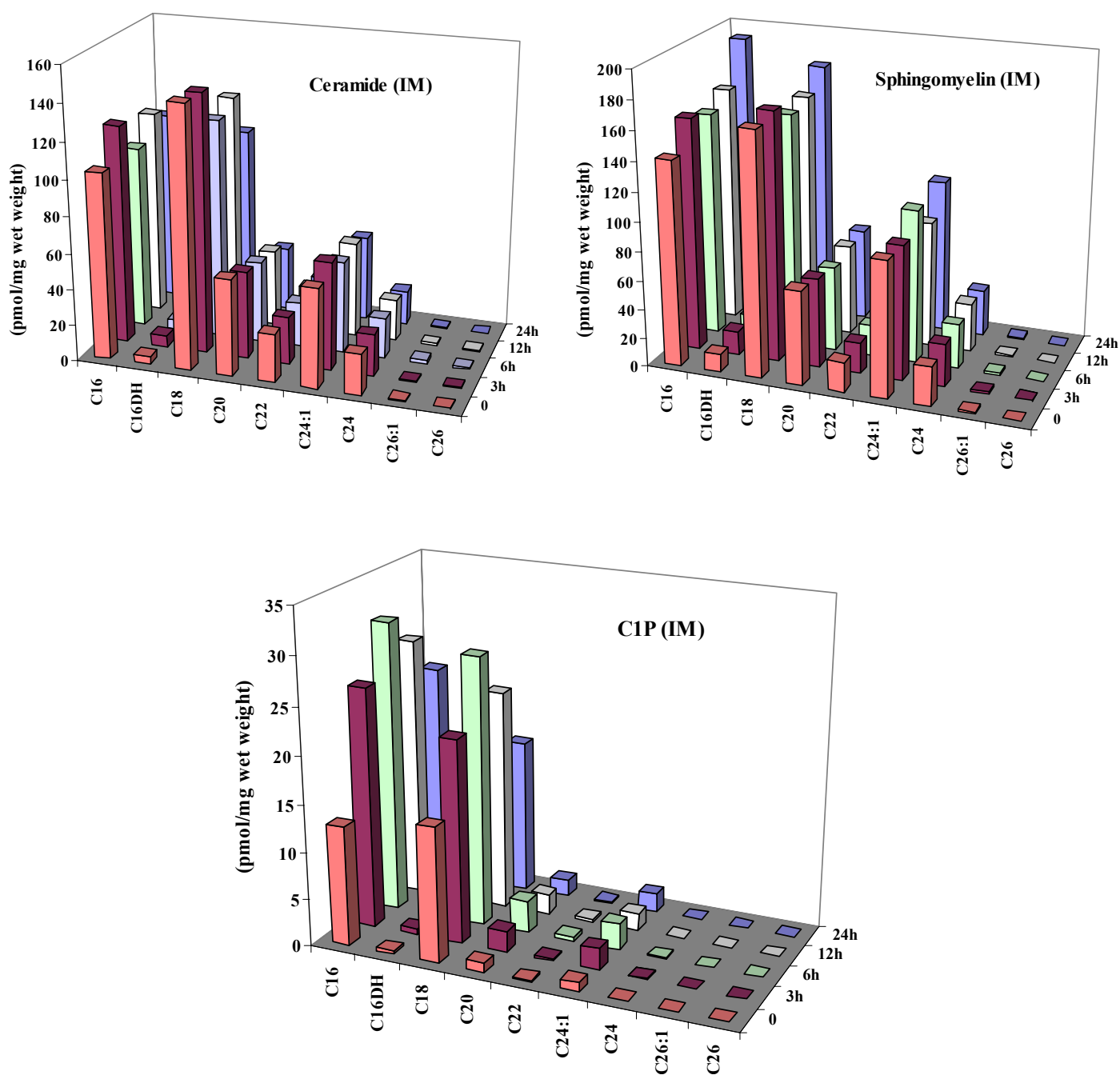
Fig. 10 Effects of intestinal manipulation (IM) on the sphingolipid content in intestinal *muscularis externa* (ME). Rat small intestine was “gently” manipulated between two moist cotton applicators (A) and the *muscularis* layer was collected at the indicated time points after surgery. Sphingolipids were extracted and analysed by HPLC-MS/MS as described in section 2.2.4. B, C, D) Data are average of triplicate determinations and are expressed as pmol of lipid/ mg wet weight in case of ceramide, sphingomyelin and sphingosine. E, F) For C1P and S1P, data are means of triplicate measurements and expressed relative to control values obtained at $t=0$. Significantly different from control values (* $p < 0.05$, ** $p < 0.01$). C1P, ceramide-1-phosphate; S1P, sphingosine-1-phosphate.

3.1.2. Sphingolipid subspecies in rat small intestine samples at different time intervals after surgical manipulation

Usually tissues contain several sphingolipid subspecies, different in the degree of saturation and length of fatty acids as well as in their sphingoid base, which can be sphingosine or dihydrosphingosine. Since HPLC-MS/MS allows for analysis of such structural variations we studied subspecies composition of ceramide, sphingomyelin and C1P at different time intervals postsurgery. HPLC-MS/MS analysis revealed no significant changes in ceramide, sphingomyelin subspecies before and after trauma in *muscularis* samples (Fig. 11 A). As illustrated in Fig. 11 A and better in “donut” diagrams (Fig. 11 B), the most abundant subspecies of these two bioactive lipids in *muscularis* layer were C16 and C18 with about 25% and 35%, followed by C24:1 and C20 with about 13 and 15%, and only 5-7% were C22 and C24, respectively with no significant change over the entire time course. By contrast, some subspecies of C1P (C16, C18, C20 and C24:1 C1P) were significantly elevated after manipulation. This difference was evident early, peaked at 6h after manipulation and decreased slowly towards control levels. Mass spectrometric analysis indicated that C16 and C18 were the prevailing C1P subspecies followed by C20 and C24:1 throughout the time course. Moreover, “donut” diagrams showed that *muscularis* layer after intestinal manipulation exhibited a larger fraction of the C16:0 fatty acid C1P subspecies *versus* C18:0

when compared to non-manipulated samples (Fig. 11 B). These results suggest that surgical manipulation induces changes not only in C1P content in rat intestinal *muscularis* tissue but obviously one certain subspecies was preferentially elevated namely C16 on the expense of C18. Although these changes are subtle, they demonstrate that manipulation of the small intestine alters both the total amount and the structure of C1P. Moreover, these data suggest that ceramide kinase uses preferentially C16 and C18 ceramides as substrates.

A



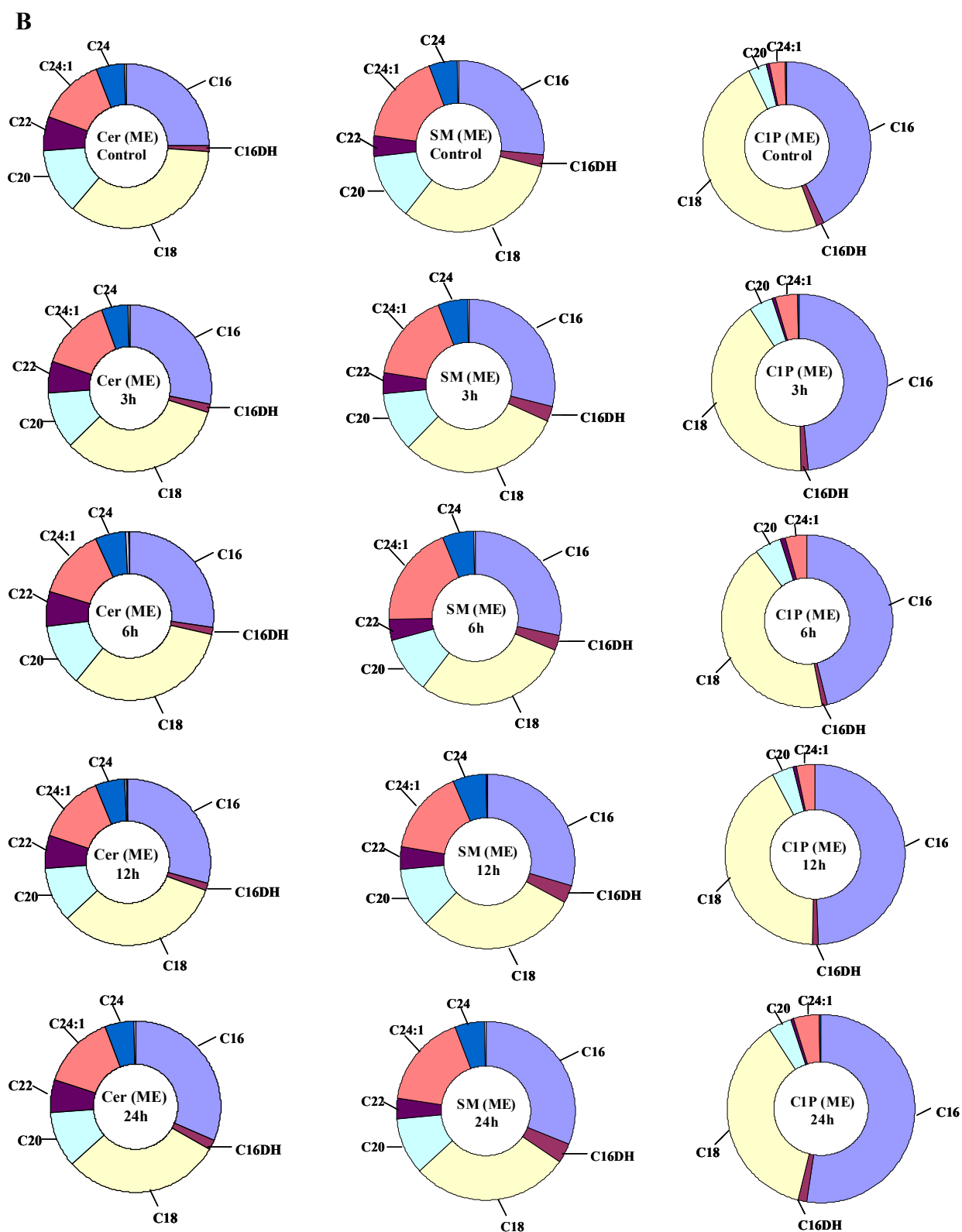


Fig. 11 Sphingolipid (ceramide, sphingomyelin, C1P) subspecies in the tunica muscularis externa (ME) after intestinal manipulation (IM). A) Rat small intestine was “gently” manipulated and the *muscularis* layer was collected at the indicated time points after surgery. Sphingolipids were extracted and the indicated species including all detectable subspecies were determined by HPLC-MS/MS as described in section 2.2.4. Data from B represent “donut” diagrams showing a proportion of each indicated sphingolipid subspecies. Data are means of triplicate determinations and are expressed as pmol of lipid/mg wet weight. *Numbers* indicate chain length followed by the number of double bonds in the fatty acid. 0 h-control; C16DH, C16 dihydroceramide; Cer, ceramide; C1P, ceramide-1-phosphate; SM, sphingomyelin.

In conclusion, lipid analysis revealed that surgical manipulation of the small intestine had no effect on ceramide content, but induced an increase of two sphingolipid-phosphates, of S1P and of C1P contents, albeit only in *muscularis* layer.

3.2. Studies with primary cultured rat intestinal smooth muscle cells

The finding that “gentle” surgical manipulation of the small bowel resulted in an increase of S1P and C1P amounts only in the intestinal *tunica muscularis externa*, prompted us to analyse the potential involvement of these two sphingolipid-phosphates in inflammatory processes in primary cultured rat intestinal smooth muscle (RISM) cells.

For this purpose, first a primary culture of rat intestinal smooth muscle cells was established. Second, we analysed the effects of S1P and C1P on various proinflammatory parameters, including, COX-2, PGE₂, cytokines (IL-1 β , IL-6) and we tried to identify the sphingolipid downstream targets involved in the inflammatory cascade. Moreover, the potential role of sphingosine kinase (SK) as a mediator of an inflammatory response was determined.

3.2.1. Characterization of rat intestinal smooth muscle (RISM) cells

Smooth muscle cells represent the major cell type in the intestinal *muscularis* layer and their contractility is primarily affected after abdominal surgery [Kalff et al., 1999a]. We have therefore established a primary culture of RISM 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 using type II collagenase and dispase II enzymes. The cells were cultured through 4-5 passages because at a higher passage of seven to eight the cells differentiate in myofibroblasts and fibroblasts. A homogeneous culture of smooth muscle cells was achieved by density gradient centrifugation. This enabled the separation from other cell types known to 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. Expression of α -actin was assessed by fluorescence microscopy and it was immunolocalized using an anti-mouse smooth muscle α -actin monoclonal antibody as primary antibody. As shown in Fig. 12 semi-confluent grown cells clearly exhibited immunoreactivity for smooth muscle α -actin, thus supporting the smooth muscle origin of our cell cultures.

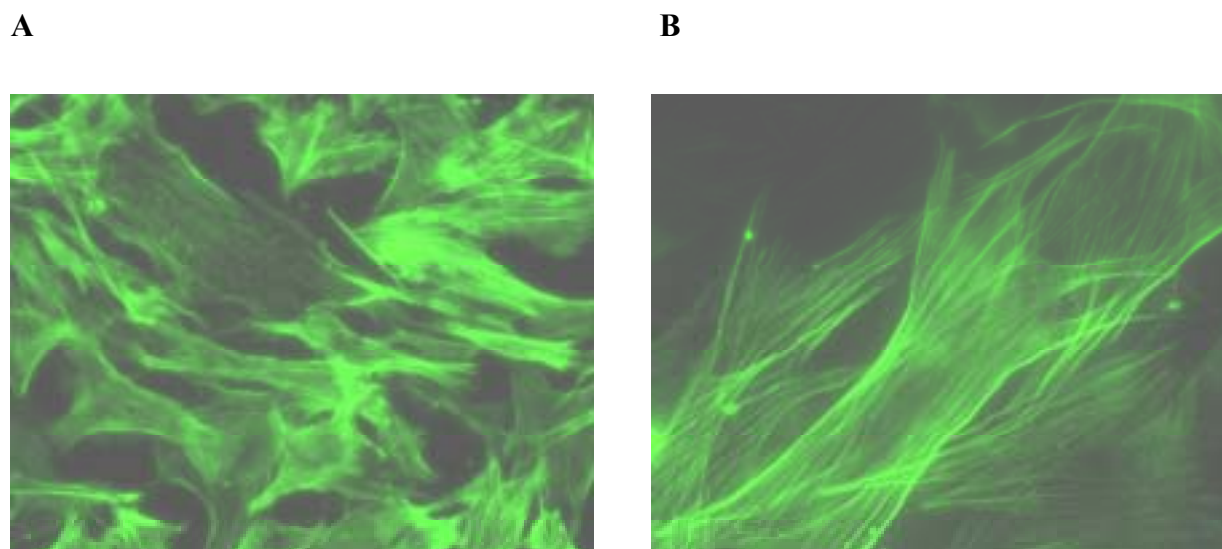


Fig. 12 Expression of smooth muscle contractile protein, α -actin in primary cultured RISM cells. Cells plated on glass coverslips were grown to 50% confluence, fixed and stained with antibody against smooth muscle α -actin isoform (1:100). Anti-mouse secondary antibody conjugated to green fluorescent Alexa Fluor 488 dye was used at 1:200. Immunoreactivity was visualized by fluorescence microscopy (40x; **A**), (63x; **B**).

3.2.2. Involvement of sphingosine-1-phosphate and ceramide-1-phosphate in inflammatory pathway in RISM cells

Next, we addressed the possible role of S1P and C1P, the sphingolipid-phosphates whose levels changed after intestinal manipulation in inflammatory cascade using primary cultured RISM cells.

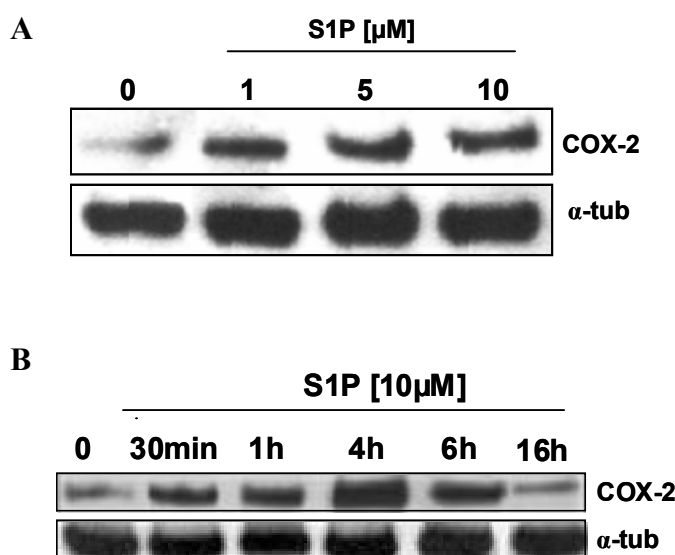
3.2.2.1. Effects of sphingosine-1-phosphate on cyclooxygenase-2 expression and prostaglandin E₂ formation in RISM cells

It has been shown on the one hand that PGE₂ formation via COX-2 is a causative mechanism of postoperative ileus [Schwarz et al., 2001]. On the other hand, S1P induced COX-2 expression and PGE₂ formation in different cell lines [Kim et al., 2003; Pettus et al., 2003b]. Therefore, in the present study we examined the expression of COX-2 protein in the presence of S1P in primary cultured RISM cells. The cells were treated for 4 h with different concentrations of S1P namely, 1 μ M, 5 μ M and 10 μ M, respectively (Fig. 13 A) or with 10 μ M of S1P for different time intervals, 0, 30 min, 1 h, 4 h, 6 h and 16 h, respectively (Fig. 13 B). Control cells were treated with the corresponding amount of methanol, the solvent used to solubilize S1P. At the respective time points, cells were harvested and lysed as described in section 2.2.7.1. The same amounts of protein (10 μ g) were separated on acrylamide gels, and analysed by immunoblotting with anti-COX-2 primary antibody and protein G-HRP-conjugated secondary antibody and detected by chemiluminescence.

As shown in Fig. 13 A, S1P induced a considerable increase of the 72-kDa COX-2 protein already at a concentration of 1 μ M with no significant change in the induction at higher concentrations. The time course of S1P-induced COX-2 expression revealed a quite fast effect, reaching a maximum that in average amounted $400\% \pm 60\%$ already after 4 h (Fig. 13 B).

Considering that PGE₂ has emerged as the prototypical eicosanoid with key function in acute inflammatory responses [Kuehl and Egan, 1980; Dempke et al., 2001], we also investigated the effect of S1P on PGE₂ formation in primary cultured RISM cells. Media were collected from cells treated for 4h with different S1P-concentration or alternatively cells were treated with 10 μ M S1P for various time intervals and assayed for secreted PGE₂ using an ELISA kit as described in section 2.2.8.1.

As shown in Fig. 13 C, S1P clearly enhanced PGE₂ formation in a concentration dependent manner ranging from 1 to 10 μ M, with little further induction from 5 to 10 μ M. Moreover, S1P induced a pronounced (about 6-fold) but transient increase in PGE₂ formation after as early as 4 h (Fig. 13 D), returning to control levels at later time intervals. Note the similarity of the time course of the effect of S1P on COX-2 expression and on PGE₂ production, respectively. Taken together, these results indicate that S1P induces COX-2 and PGE₂ production in primary cultured RISM cells.



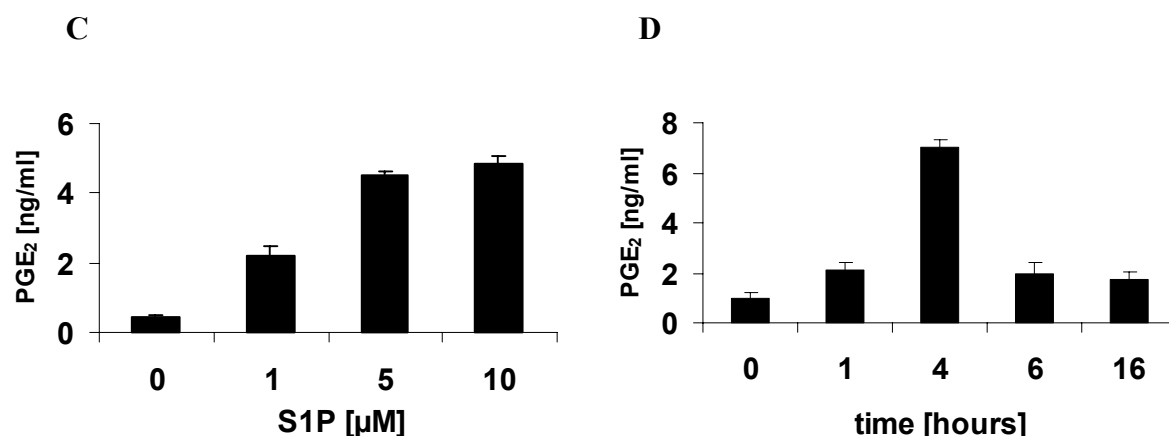


Fig. 13 Effects of S1P on COX-2 induction and PGE₂ synthesis in primary cultured RISM cells. A) Cells were incubated with the indicated concentration of S1P for 4 h or B) with 10 μM S1P for the indicated times. Control (time 0) cells were always treated accordingly in the presence of vehicle. Lysed cell protein (10 μg) was subjected to 10% SDS-PAGE and immunoblot analysis to determine COX-2 expression as described in section 2.2.7.4. An anti- α -tubulin (α -tub) antibody was used to confirm equal loading. The blots shown are from one representative out of at least three independent experiments. C, D) Media from cells treated as mentioned above (in A and B) were collected and subjected to PGE₂ analysis as described in section 2.2.8.1. Data are means \pm S.D. from three independent experiments

3.2.2.2. Intracellular signalling pathway of sphingosine-1-phosphate-induced cyclooxygenase-2 expression in RISM cells

It is well known that S1P can act either as an extracellular ligand by binding to a family of G-protein coupled receptors (GPCRs) or as an intracellular second messenger with so far unknown direct targets [Spiegel and Milstien, 2003]. All five members of the S1P-receptor family can be coupled to G_{i/o}-proteins [Ishii et al., 2004]. Since the latter are inactivated by pertussis toxin (PTX) via ADP-ribosylation, this toxin was used to find out in a first approach whether the stimulatory effect of S1P on COX-2 expression is receptor-mediated. Thus, primary cultured RISM cells were preincubated with 100 ng/ml of PTX prior to stimulation with 10 μM S1P, shown previously to up-regulate COX-2. As illustrated in Fig. 14, pre-treatment with PTX abrogates S1P-induced COX-2 expression. Furthermore, PTX-sensitivity indicates the involvement of a G_i-coupled S1P receptor in COX-2 expression. However, the considerable stimulation of COX-2 expression by PTX alone suggests the complexity of COX-2 expression. Of interest, S1P₂ receptor was identified by RT-PCR in RISM cells (data not shown).

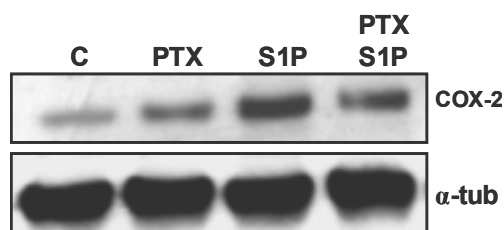


Fig. 14 Effects of PTX on S1P-induced COX-2 expression in primary cultured RISM cells. Cells were preincubated in the absence or presence of 100 ng/ml PTX for 2 h prior to treatment with 10 μ M S1P for 4h. Controls (C) were treated accordingly using vehicle. Each sample (10 μ g of protein) was subjected to 10% SDS-PAGE, and the expression levels of COX-2 were determined by immunoblot analysis using anti-COX-2 antibody. The blot shown is one representative out of at least three independent experiments.

S1P receptor-mediated effects often imply MAPK signalling pathways, some of them being implicated in the regulation of COX-2 expression in different cell types [Kim et al., 2003; Damirin et al., 2005]. In this study, we therefore investigated whether S1P stimulates MAPKs using Western blot analysis with anti-phospho-specific antibodies against each enzyme. To confirm equal amount of protein loaded for each sample, blots were subsequently stripped and reprobed with an antibody against total ERK MAPK and total p38 MAPK, which are constitutively expressed in all cells at high levels. In cells treated with 10 μ M S1P for different time intervals (15 min, 30 min, 1 h, 4 h and 16 h, respectively), the level of phosphorylated ERK was transiently increased, showing maximal activity within 30 min up to 1 h of treatment (Fig. 15 A) and then decreased slowly at 4h after incubation with S1P. p38 MAPK was also transiently activated by S1P treatment (Fig. 15 B). By contrast, JNK was insensitive to S1P administration (Fig. 15 C). These results indicate that S1P activates both, ERK MAPK and p38 MAPK, in primary cultured RISM cells.

To determine a potential correlation of MAPK phosphorylation and S1P-induced COX-2 expression, we used two pharmacological MAPK inhibitors. Cells were preincubated with PD-98059 a specific inhibitor for ERK MAPK [Dudley et al., 1995] or alternatively with SB 239063 [Underwood et al., 2000] a p38 MAPK inhibitor, prior to administration of 10 μ M S1P for 4 h. The same amounts of protein (10 μ g) were separated on acrylamide gels, and analysed by immunoblotting with anti-COX-2 primary antibody and protein G-HRP-conjugated secondary antibody followed by chemiluminescence. The preincubation of RISM cells with 5 μ M of SB 239063, a specific p38 MAPK inhibitor, prior to S1P treatment completely inhibited COX-2 expression by S1P (Fig. 15 D). Similar results were obtained with 30 μ M PD-98059, known to specifically inhibit ERK MAPK [Natzker et al., 2002] (Fig. 15 D). These results indicate that both, ERK MAPK and p38 MAPK are essentially involved in S1P-induced COX-2 expression.

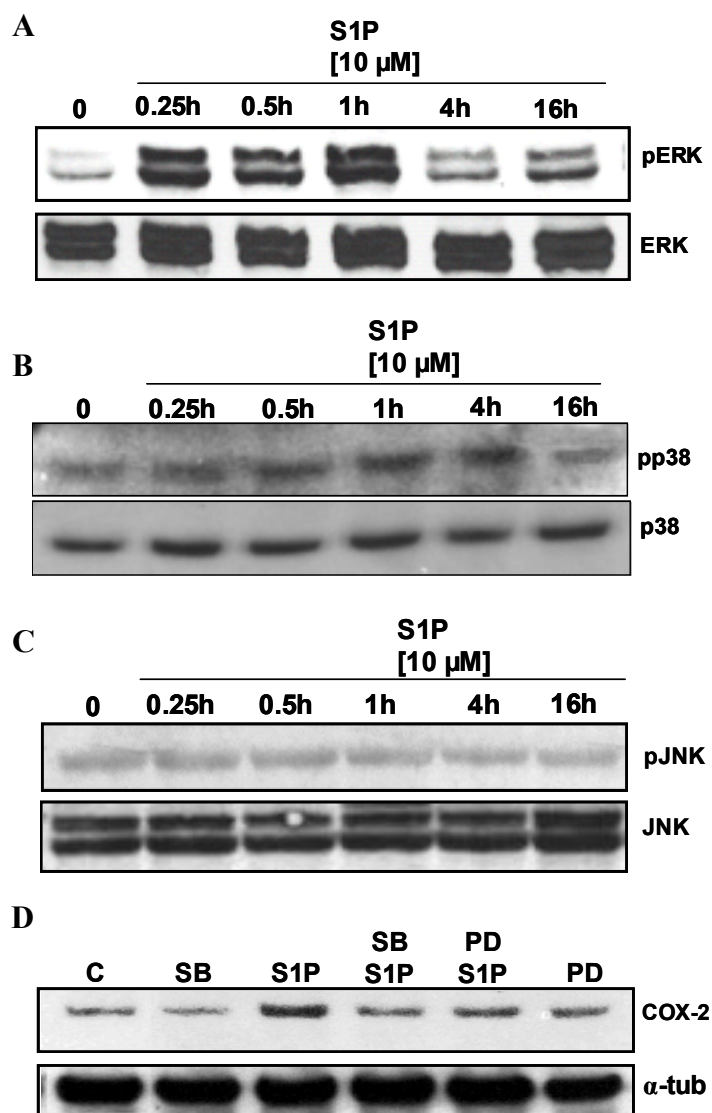


Fig. 15 Effect of MAPKs on COX-2 expression in primary cultured RISM cells. **A, B, C)** Cells were stimulated with 10 μ M S1P for various time intervals. At the respective time points, the cells were harvested and lysed as described in section 2.2.7.1. Each sample (20 μ g of protein) was subjected to 10% SDS-PAGE and phosphorylated ERK (pERK), phosphorylated p38 MAPK (pp38) or phosphorylated JNK (pJNK) were determined by immunoblotting with a specific antibody, respectively. Blots were stripped and reprobed with an antibody specific for total ERK, p38 MAPK or total JNK, respectively, to confirm equal loading. **D)** Cells were pretreated with vehicle, 5 μ M SB-239063 or 30 μ M PD-98059 for 30 min prior to incubation with 10 μ M S1P. After 4 h cells were lysed and 10 μ g of protein was subjected to 10% SDS-PAGE and COX-2 expression level was determined by immunoblot analysis using anti-COX-2 antibody. An anti- α -tubulin (α -tub) antibody was used to confirm equal loading. The results shown are one representative out of at least three independent experiments (A-D).

3.2.2.3. Effects of sphingosine-1-phosphate on cytokine production in RISM cells

It has been recently shown that intestinal surgical manipulation is accompanied by induction of the proinflammatory cytokine IL-6 within the *tunica muscularis externa* of rodent small intestine [Wehner et al., 2005]. Moreover, it was reported that S1P induces substantial IL-6 secretion in human airway smooth muscle cells, thus indicating that S1P is an important inflammatory mediator implicated in the pathogenesis of asthma [Ammit et al., 2001].

To explore the potential role of S1P in inflammatory responses that accompany postoperative ileus, we examined the effects of exogenous S1P on cytokine expression in primary cultured RISM cells. Cells were treated with 5 and 10 μM of S1P, respectively, for 4 h (Fig. 16 A) or with 10 μM of S1P for different time intervals (1 h, 4 h and 6 h) (Fig. 16 B). Control cells were treated with the corresponding amount of methanol, the solvent used to solubilize S1P. At the respective time points, media were collected and subjected to ELISA assay to determine the concentration of IL-6 and IL-1 β , respectively. As shown in Fig. 16 A, S1P-induced IL-6 production increased 4 h post-treatment with 5 and 10 μM S1P by ($486,7 \pm 40$ pg/ml) and (650 ± 23 pg/ml), respectively, compared to controls that amounted 260 ± 70 pg/ml). The time course of IL-6 secretion by cells treated with 10 μM S1P revealed a maximal increase of IL-6 production at 4 h ($200\% \pm 20\%$) (Fig. 16 B). S1P also induced an up-regulation of IL-6 at transcriptional level in a concentration-dependent manner. Maximal induction of transcription was obtained after 4 h treatment with 10 μM of S1P (Broere, personal communication). Moreover, S1P stimulated IL-6 secretion in a PTX-sensitive manner (not shown), suggesting that the effects of S1P on IL-6 expression are mediated via S1P receptors. In contrast to its observed effects on IL-6 secretion S1P did not induce expression of IL-1 β , neither on protein level (Fig. 16 C), nor on transcriptional level (data not shown). These results indicate that exogenous S1P can regulate the production of IL-6 in primary cultured RISM cells, suggesting one more time the possible implication of S1P in inflammatory processes that go along with postoperative ileus.

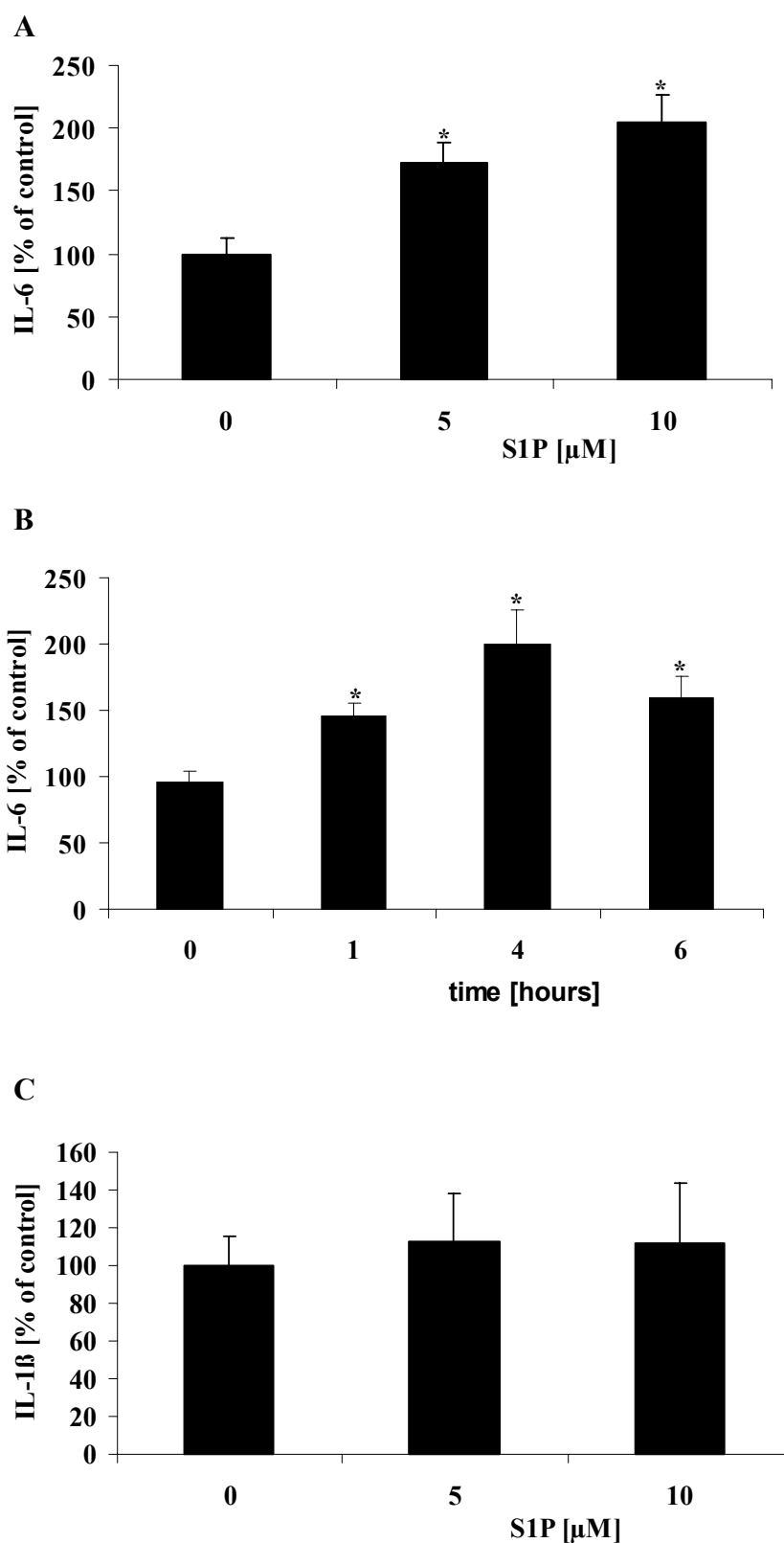


Fig. 16 Effects of S1P on cytokine secretion in primary cultured RISM cells. A) Cells were incubated with the indicated concentrations of S1P for 4 h or B) with 10 μ M of S1P for the indicated times. Media from all samples were collected and IL-6 (A, B) or IL-1 β (C) was measured by ELISA assay as described in section 2.2.8.2. Data are means \pm S.D. of three independent experiments. * $p < 0.05$ significant different from controls.

3.2.2.3.1. Involvement of sphingosine kinase in TNF- α -induced IL-6 cytokine production in RISM cells

The finding that S1P induces production of the proinflammatory mediator IL-6 in primary cultured RISM cells, prompted us to investigate if sphingosine kinase (SK), which generates S1P, may function as an endogenous regulator of IL-6 secretion in response to TNF- α . Previous studies have shown that TNF- α induces a transient increase in S1P level in different cell lines [Pettus et al., 2003b]. Moreover, it was reported that SK1 activation is involved in the signal transduction pathway initiated by TNF- α in A549 cells [Billich et al., 2005]. Therefore, we explored whether or not SK is involved in IL-6 production in response to TNF- α . Note that the latter is a pleiotropic cytokine whose secretion increases after intestinal manipulation of the rat small bowel (Speidel N, personal communication).

To determine the potential role of SK as a mediator of TNF-induced IL-6 elevation, cells were stimulated for 4 h with TNF- α (10 ng/ml) in the absence or presence of 10 μ M of N, N-dimethylsphingosine (DMS) a potent inhibitor of SK (Endo K, 1991). IL-6 was determined as described in section 2.2.8.2 in the cell culture medium, after removal of detached cells by centrifugation using the respective ELISA. We observed that TNF- α induced an elevated IL-6 secretion in primary cultured RISM cells (2-fold after 4 h treatment) (Fig. 17 A). As shown in Fig.17 A, DMS was capable to suppress production of IL-6 following stimulation of cells with TNF- α suggesting that SK activation is required for expression of IL-6. Furthermore, we measured the enzymatic activity of SK after treatment with TNF- α for 4 h. As becomes clear from Fig. 17 B, TNF- α induces an increase of SK enzyme activity by $191\% \pm 19\%$. Note also the generation of C1P indicating a simultaneous activation of ceramide kinase (CK) by TNF- α (Fig.17 B, TLC). It can, however, not be excluded that in these cells SK does not exhibit substrate specificity, thus phosphorylating both, sphingosine and ceramide following TNF- α treatment. Taken together these results suggest that TNF-induced IL-6 secretion is dependent of SK and might also involve CK.

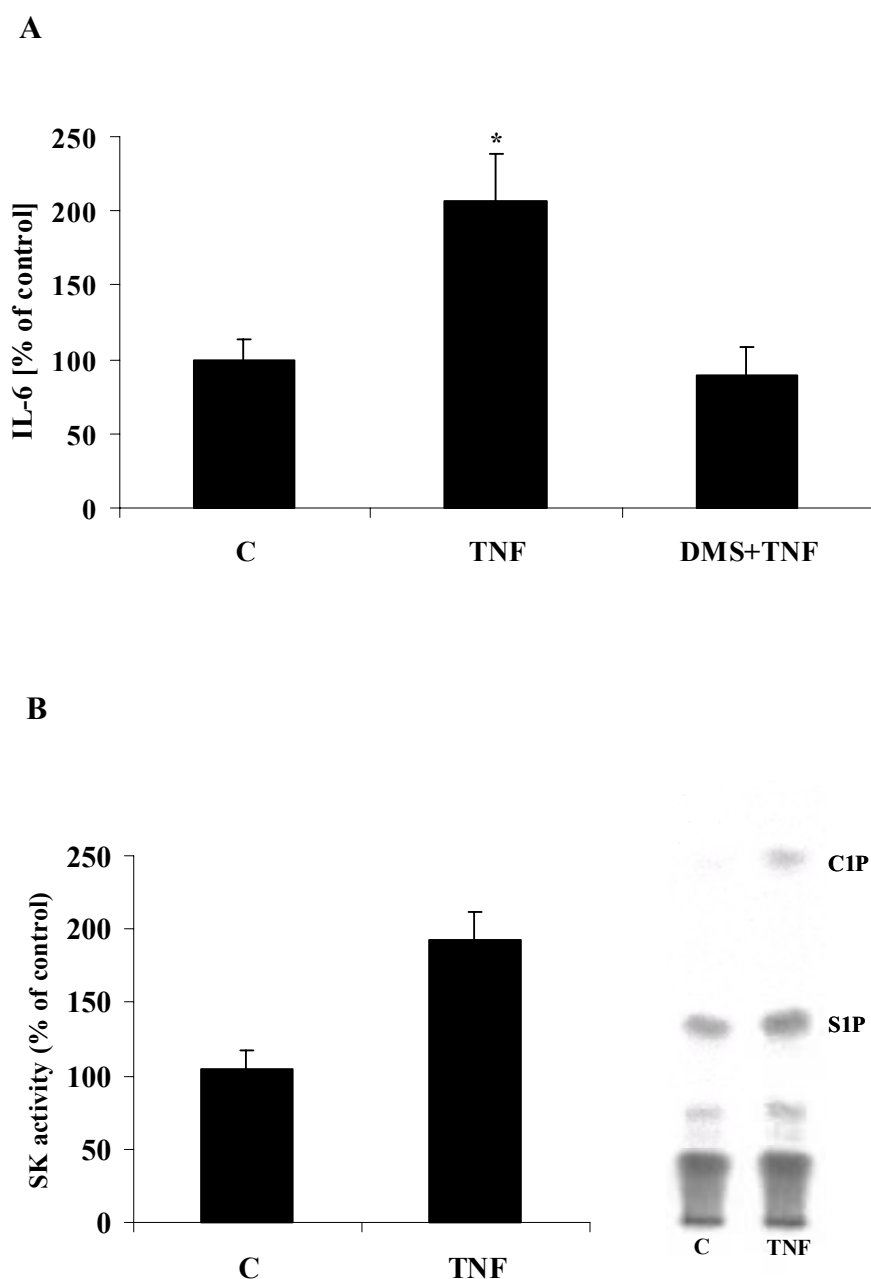


Fig. 17 Involvement of SK in TNF-induced IL-6 secretion in primary cultured RISM cells. **A)** Cells were incubated with TNF- α (10 ng/ml) in the absence or presence of DMS (10 μ M) for 4 h. Control cells (C) were always treated accordingly in the presence of vehicle. Media were collected and subjected to ELISA assay as described in section 2.2.8.2. Data are means \pm S.D. from three independent experiments. * $p < 0.05$ treated with TNF vs. control cells **B).** Cells were incubated for 4h with vehicle (C) or with 10 ng/ml TNF- α . After 4 h, cells were harvested and sphingosine kinase activity was measured in cell homogenates using selective buffer conditions as described in section 2.2.5. 32 P-labelled lipids were resolved by TLC with 1-butanol/methanol/acetic acid/water (80:20:10:20) and visualized by autoradiography using the bio-imaging analyzer Fujix bas 1000 software, Tina 2.09. The radioactive spots corresponding to S1P and C1P were identified using S1P and C1P as standards. Data are means \pm SD from two independent experiments, each with double determination.

3.2.2.4. Effects of ceramide-1-phosphate on cyclooxygenase-2 expression and prostaglandin E₂ formation in RISM cells

Although, ceramide-1-phosphate (C1P) was identified more than a decade ago, its potential biological functions have been reported only in the last few years [Liang et al., 2003; Pettus et al., 2003a]. In recent studies, C1P has emerged as a key regulator of inflammatory responses [Pettus et al., 2003a; Pettus et al., 2004a]. Our studies on rat small intestine indicated an increase in the amount of C1P in *muscularis* layer following surgical manipulation. Therefore, we addressed the potential involvement of this sphingolipid-phosphate in inflammatory processes in primary cultured RISM cells.

First, we examined its effect on the expression of COX-2 protein using S1P as a positive control. The cells were treated with 10 μ M of C1P and S1P, respectively. Control cells were treated with the corresponding amount of methanol, the solvent used to solubilize C1P and S1P. After 4 h, cells were harvested and lysed as described in section 2.2.7.1. The same amounts of protein (10 μ g) were separated on acrylamide gels, analysed by immunoblotting with anti-COX-2 primary antibody and protein G-HRP-conjugated secondary antibody and detected by chemiluminescence. As shown in Fig. 18 A, C1P did not affect COX-2 expression, whereas a considerable increase of the 72-kDa COX-2 protein has been observed in the presence of S1P (see also section 3.3.2.1.).

We next analysed the effect of C1P on PGE₂ formation in primary cultured RISM cells. Media from the cells used for COX-2 detection were collected and assayed for PGE₂ secretion by ELISA. After 4 h, C1P induced a significant elevation of PGE₂ production which was however, clearly below that obtained in the presence of S1P, at least at this time point (Fig. 18 B). These results implicate the action of C1P in the pathway of PGE₂ formation but at a different step than S1P.

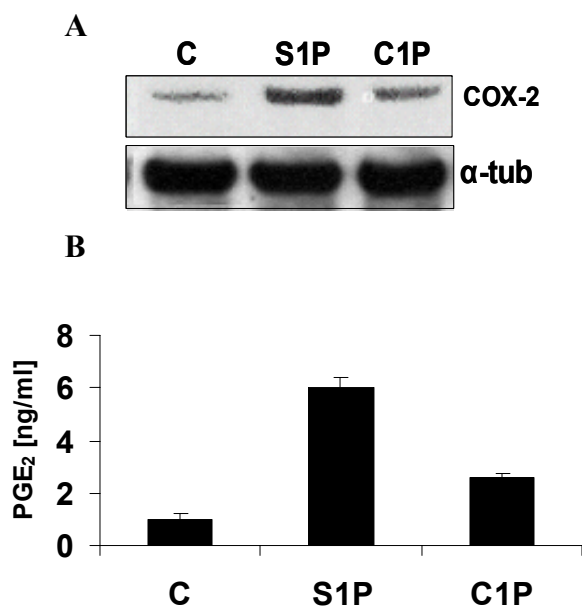


Fig. 18 Effects of C1P on COX-2 expression and PGE₂ formation in primary cultured RISM cells. **A)** Cells were incubated with 10 μ M S1P and C1P, respectively for 4 h. Control cells (C) were always treated accordingly in the presence of vehicle. **B)** Media from samples mentioned above were collected and subjected to PGE₂ analysis as described in section 2.2.8.1. Data are means \pm S.D. from three independent experiments. Lysed cell protein (10 μ g) was subjected to 10% SDS-PAGE and immunoblot analysis to determine COX-2 expression as described in 2.2.7.4. An anti- α -tubulin (α -tub) antibody was used to confirm equal loading. The blots shown are from one representative out of at least three independent experiments.

3.2.2.4.1. Effects of ceramide-1-phosphate on arachidonic acid release and prostaglandin E₂ formation in RISM cells

Since C1P clearly stimulated PGE₂ production but had no effect on COX-2 expression, we investigated its effect on arachidonic acid release, which is indicative for an enhanced PLA₂ activity. PLA₂ catalyses liberation of arachidonic acid from membrane phospholipids, which represents the initial and rate-limiting step in PGE₂ synthesis [Pettus et al., 2004b].

For this purpose RISM cells were labelled overnight with [¹⁴C]-arachidonic acid and afterwards incubated with 10 μ M of C1P for different time intervals (1 h, 4 h and 24 h, respectively). Control cells were treated with the corresponding amount of methanol, the solvent used to solubilize C1P. Following incubation, the medium was removed and an aliquot was submitted to scintillation counting to determine the amount of [¹⁴C] arachidonic acid released after treatment with C1P. As shown in Fig. 19 A, in the presence of C1P, the amount of arachidonic acid released from the cells increased in a time-dependent manner up to 3-fold at 24 h when compared to control cells. As determined by TLC, the data shown in Fig. 19 A refer only to arachidonic acid and not to arachidonic acid-labelled lipids. An aliquot of medium was used to determine the amount of PGE₂ released in the presence of C1P at different time intervals. As obvious in Fig. 19 B, in the presence of C1P the synthesis of PGE₂

ascended continuously up to 2.5-fold at 24 h. Taken together, these data indicate that C1P induces a time-dependent increase in both arachidonic acid and PGE₂ release.

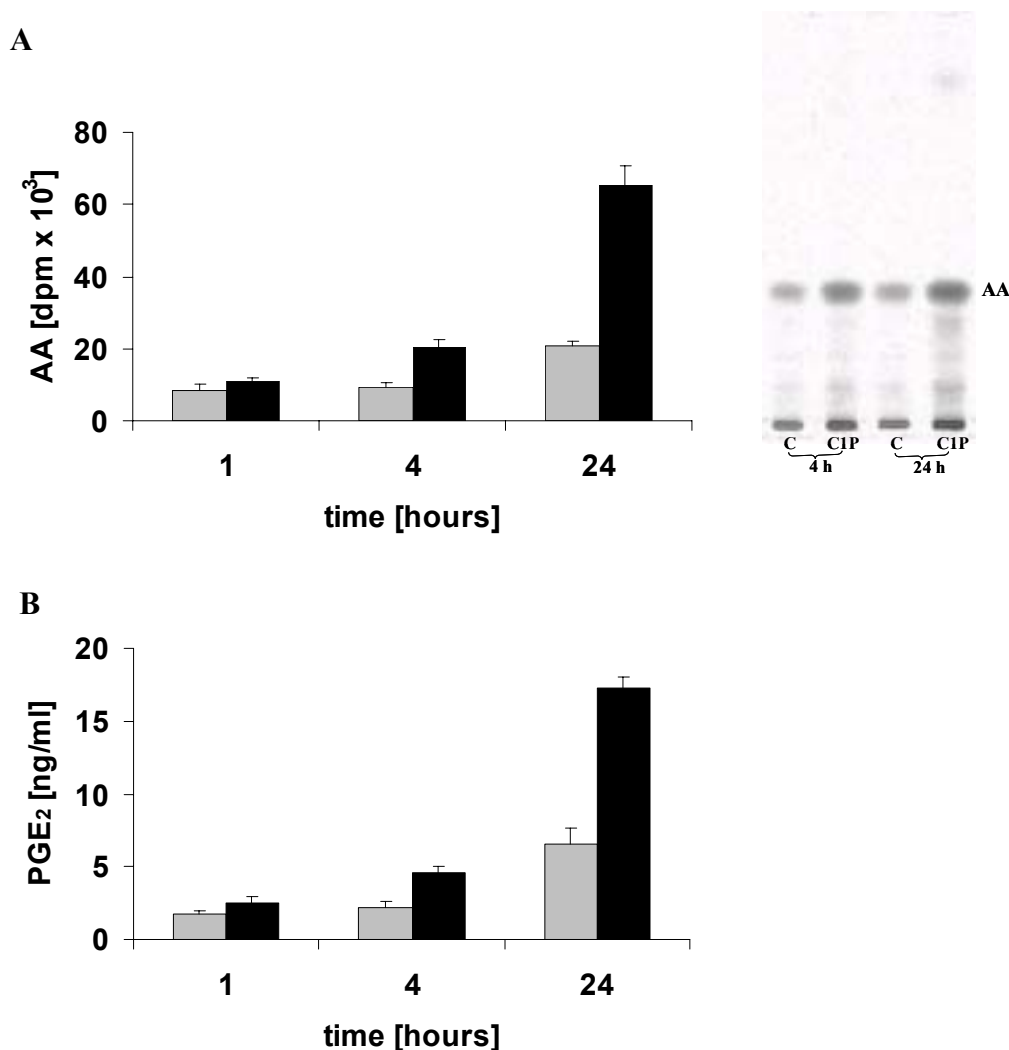


Fig. 19 Effects of C1P on arachidonic acid release and PGE₂ formation in primary cultured RISM cells. **A)** Cells were labelled overnight with 0.2 $\mu\text{Ci/ml}$ [¹⁴C] arachidonic acid (AA) (3.7 μM). Then medium was removed and cells were rinsed three times with culture medium prior to incubation in the presence of vehicle (control, grey columns) or of 10 μM C1P (black columns) for the indicated times. Results were normalized to cell protein levels. The results are presented as dpm of [¹⁴C] arachidonic acid/ml medium. In addition, media were subjected to reversed phase chromatography as described above (see section 2.2.3.4). The samples were applied on Silicagel-60 TLC-plates developed with n-hexane/diethyl ether/glacial acetic acid (70:30:1, by volume). Arachidonic acid was visualized by autoradiography using the bio-imaging analyzer Fujix bas 1000 software, Tina 2.09. **B)** In parallel experiments, the synthesis of PGE₂ was assayed as described in section 2.2.8.1. Data are means \pm S.D. of three independent experiments. C, controls.

To examine whether the effect of C1P on arachidonic acid release was specific, RISM cells were treated with closely related lipids such as, didecanoylphosphatidic acid (PA), ceramide (Cer) and also with C1P for 4 h and 24 h, respectively. Only C1P induced significant arachidonic acid release (Fig. 20) when compared with control cells. Thus, the effect of C1P on arachidonic acid release in RISM cells is highly specific.

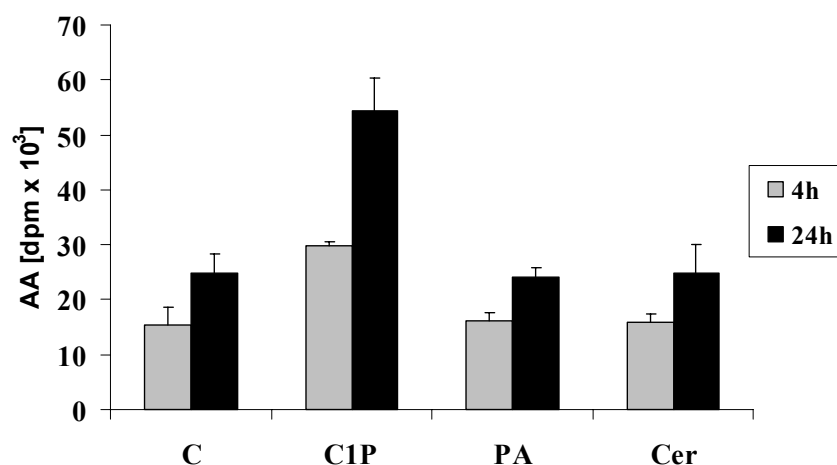


Fig. 20 Effects of C1P-related lipids on arachidonic acid release in primary cultured RISM cells. Cells were labelled overnight with 0,2 $\mu\text{Ci/ml}$ [^{14}C] arachidonic acid (AA) (3,7 μM). Subsequently, medium was removed and the cells were rinsed three times with culture medium prior to incubation in the presence of vehicle (control, C) or of 10 μM of C1P, didecanoylphosphatidic acid (PA), ceramide (Cer), for 4 h (grey columns) or 24 h (black columns). Results were normalized to cell protein levels. The results are presented as dpm of [^{14}C] arachidonic acid/ml medium. Data are means \pm S.D. of three independent experiments.

3.3. Activity of sphingosine kinase after mechanical stress of RISM cells

The data obtained with primary cultured RISM cells suggest that S1P and C1P have proinflammatory effects in these cells. Moreover, they support the hypothesis that sphingolipids might be involved in inflammatory responses occurring in the *muscularis* layer after surgical manipulation of the small intestine. To find out whether mechanical stress supposed to be causative for the inflammatory processes of intestinal *muscularis* is correlated with increased levels of S1P and C1P, cells were subjected to a mechanical stretch to mimic the insult that has been reported to induce postoperative ileus. Cells were seeded into collagen-coated silicone elastomer-base culture plates and subjected to stretching as described in section 2.2.2.6. At 30 min and 4 h post-stretching, cells were harvested and the enzymatic activity of sphingosine kinase (SK) was measured. As shown in Fig. 21, an induction of SK activity occurred already at 30 min postmechanical stretch as assessed by the quantification of its product, S1P. Interestingly, the content of C1P was also enhanced after mechanical stretch (Fig. 21, TLC) suggesting activation of ceramide kinase in response to manipulation.

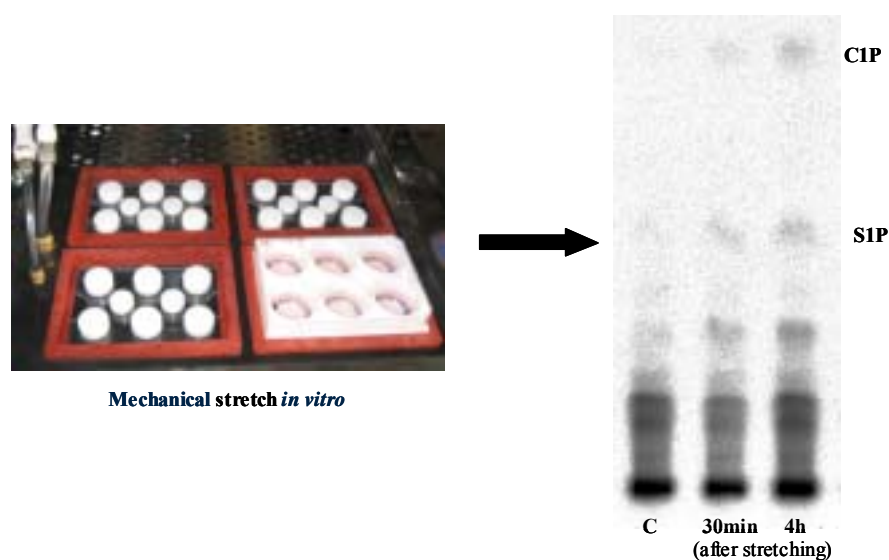


Fig. 21 Effect of mechanical stretch on SK activity in RISM cells. Cells were serum-deprived for 24h and then exposed to mechanical stretch for 2 x 30 min with 5 min break in between. At the indicated time intervals after stretching, cells were harvested and sphingosine kinase activity was measured in cell homogenates using selective buffer conditions as described in section 2.2.5. ^{32}P -labelled lipids were resolved by TLC with 1-butanol/methanol/acetic acid/water (80:20:10:20) and visualized by autoradiography using the bio-imaging analyzer Fujix bas 1000 software, Tina 2.09. The radioactive spots corresponding to authentic S1P and C1P were identified using S1P and C1P as standards. The chromatogram shown is one representative out of three independent experiments.

3.4. “In vivo” induced endotoxemia

Previous studies have shown that systemically administrated living bacteria or endotoxin have a detrimental effect on metabolic and barrier functions of the small intestine and thus promotes the translocation of bacteria from the gut lumen to the portal or systemic circulation [Swank and Deitch, 1996]. It has been shown that a single, sublethal dose of bacterial lipopolysaccharide (LPS) disrupts *in vivo* and *in vitro* gastrointestinal motility and transit [Weisbrodt et al., 1996; Eskandari et al., 1997; Cullen et al., 1999b]. Moreover, it has been demonstrated that LPS causes ileus by initiating an inflammatory response within the intestinal *muscularis* layer [Eskandari et al., 1997] with an excess production of inflammatory mediators [Turler et al., 2002].

To analyse the potential involvement of sphingolipids in LPS-induced ileus, we used an *in vivo* standard rodent model of endotoxemia. Endotoxemia was induced by a single intraperitoneal injection of LPS (10 mg/kg). As a result of LPS administration, the levels of sphingolipids in intestinal tissue might be altered. We therefore analysed the sphingolipid composition of the rat small intestinal tissue (*mucosa* and *muscularis* layers, respectively) at different time intervals i.e. 0 h, 3 h, 6 h, 12 h and 24 h after LPS exposure. Several studies are suggesting a role of bioactive sphingolipids in LPS signalling [Haimovitz-Friedman et al., 1997; Memon et al., 1999; Wu et al., 2004].

3.4.1. The content of sphingolipids in rat small intestine samples at different time intervals after lipopolysaccharide administration

3.4.1.1. Analysis of sphingolipids by thin layer chromatography

To analyse the effect of LPS on sphingolipid composition in rat small intestine, *mucosa* and *muscularis* layers (50 mg and 25 mg wet weight, respectively) were collected at various time intervals (0 h, 3 h, 6 h, 12 h and 24 h) following LPS injection (10 mg/kg) and subjected to lipid analysis. Lipids were extracted and analysed by TLC as described in 2.2.3. Much attention was focused on ceramide level, the primary sphingolipid found to play a role in inflammatory processes. To statistically analyse the level of sphingolipids in rat small intestine after LPS injection, three groups of samples were prepared and processed in independent experiments. For each sample, ceramide content was expressed as the percentage of total lipids extracted. Ceramide was detected in both, rat intestinal *mucosa* (Fig. 22 A) and *muscularis* (Fig. 22 B) layers, amounted about 4% and 3%, respectively of total lipids. The results showed no significant change neither in the level of ceramide (Fig. 22 C) nor of other

sphingolipids (not shown) at any time point after LPS injection when compared with 0 h, i.e. non-treated controls in both layers.

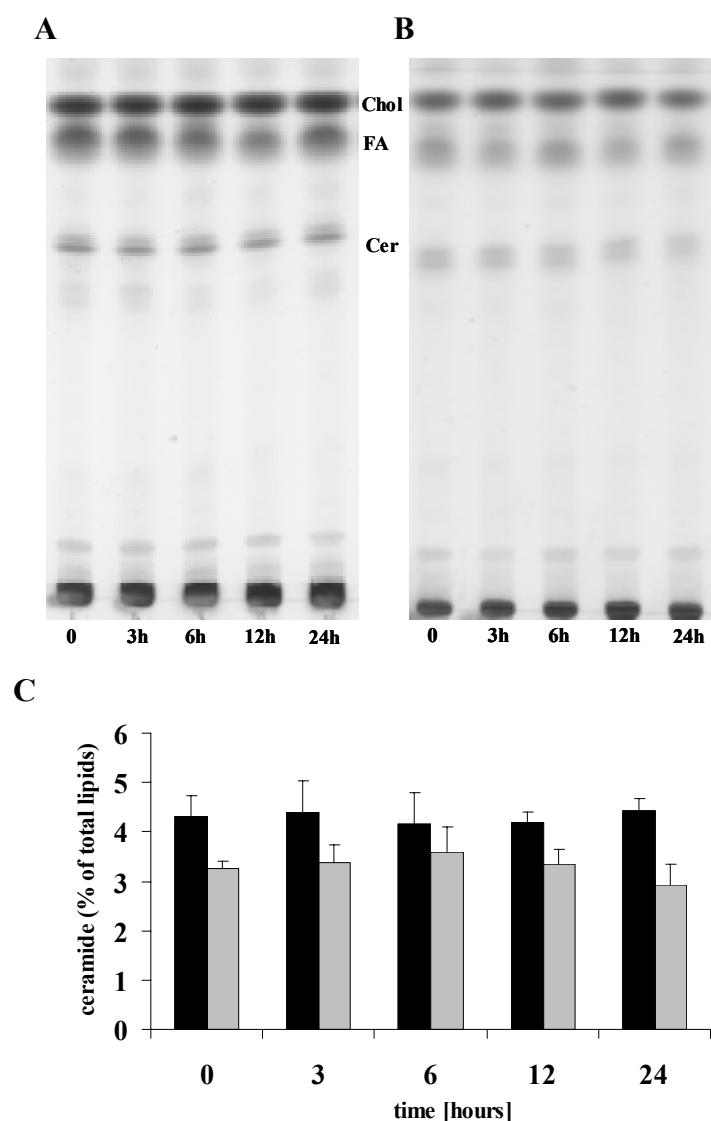


Fig. 22 Effect of LPS treatment on ceramide content in intestinal *mucosa* (MC) and *muscularis* (ME). Rat intestinal *mucosa* (A) and *muscularis* (B) were collected at indicated time points after LPS administration. The specimens were homogenized in 600 μ l water and lipids were extracted with chloroform/methanol (1:2,v/v). After mild alkaline hydrolysis to remove glycerophospholipids, lipid extracts were desalted and subjected to TLC as described in sections 2.2.3.4, 2.2.3.6. In some experiments lipid extracts were subjected to anionic exchange chromatography. The TLC plate was run twice consecutively in chloroform/ methanol/acetic acid (190:9:1; v/v/v). For detection of lipids the TLC was sprayed with 8% phosphoric acid/ 10% cupric sulphate and developed for 10 min at 185°C. The mobility of standard lipids is indicated. C) Ceramide (black columns, *mucosa*; grey columns, *muscularis*) was quantified by photodensitometry. Data are averages of three independent experiments. Cer, ceramide; FA, fatty acid; Chol, Cholesterol.

3.4.1.2. Analysis of sphingolipids by mass spectrometry

To confirm the results obtained by TLC analysis and to detect sphingolipids, which are normally found in very low amounts in biological materials we employed high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). 50 mg wet weight of *mucosa* layer and 30 mg wet weight of *muscularis* layer from control and LPS-treated animals (0 h, 3 h, 6 h, 12 h and 24 h) respectively, were subjected to lipid analysis by HPLC-MS/MS. No significant change in the amount of ceramide, sphingomyelin and sphingosine was detected at any time interval after LPS injection in comparison with controls, non-treated rat intestine samples, in both, *mucosa* and *muscularis* layers. In contrast, the amounts of C1P and S1P were significantly affected by LPS treatment, intestinal *muscularis* exhibiting a time dependent increase in the content of C1P and S1P (Fig. 23 A, B). C1P and S1P ranged in a picomolar region, no significant changes of any of these two sphingolipid-phosphates being observed in the *mucosa* layer after LPS injection (data not shown). The content profiles of the two sphingolipid-phosphates were quite similar. Both peaked at 12 h after injection, reaching a more than 3-fold higher level at this time point when compared with non-treated rat intestinal samples. After 12 h the level of both, decreased towards control levels (Fig. 23 A, B). Note that a significant increase in S1P content occurred even at 6 h post-treatment.

These results indicate that C1P and S1P are the only sphingolipids of the investigated ones that could play a role in sepsis-induced ileus. The findings that intestinal manipulation induced likewise an increase in the contents of C1P and S1P support the hypothesis that both, LPS- and postoperative induced-ileus share a number of similar pathways [Eskandari et al., 1997; Kalff et al., 1998]. Moreover, these findings might indicate for the first time a direct involvement of these two bioactive sphingolipids in LPS signalling in an animal model.

We have also examined the effects of LPS on sphingolipid (ceramide, sphingomyelin, C1P) subspecies measured by HPLC-MS/MS. HPLC-MS/MS analysis revealed similar results obtained after intestinal manipulation with no significant changes in ceramide, sphingomyelin subspecies before and after LPS administration. Likewise, the most abundant subspecies of these two bioactive lipids in *muscularis* layer over the entire time course were C16 and C18 followed by C24:1 and C20 and C22 and C24, respectively with no significant change at any time point post-treatment. By contrast, some subspecies of C1P (C16, C18, C20 and C24-C1P) were significantly elevated after LPS injection. This difference was evident early, peaked at 12 h after LPS administration and then decreased slowly towards control values.

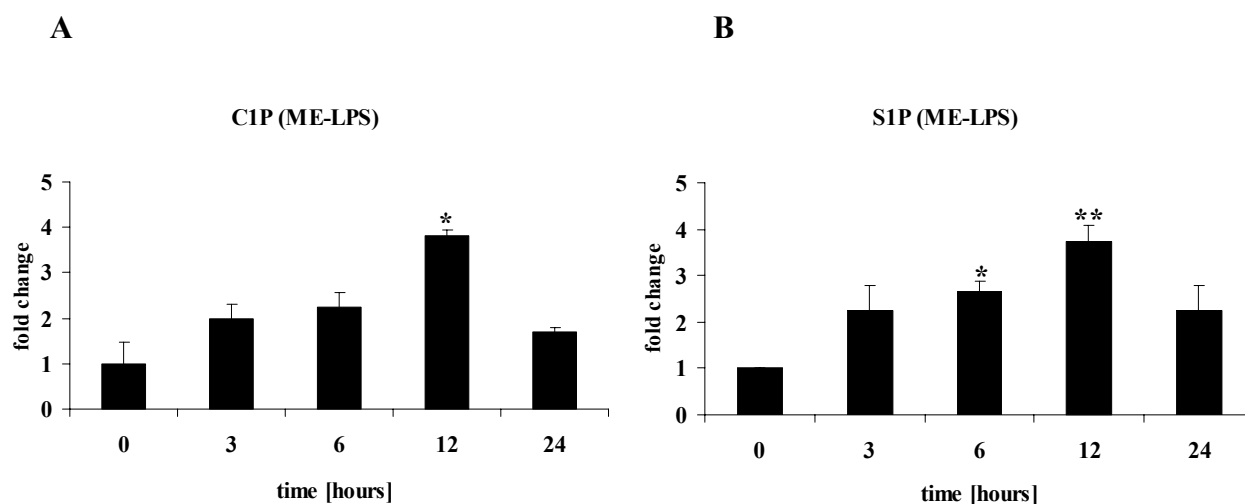


Fig. 23 Effects of LPS on C1P and S1P content in rat intestinal *muscularis* (ME). Rats were injected intraperitoneally with LPS (10 mg/kg) and sacrificed at various time points (0h, 3 h, 6h, 12 h and 24 h) after treatment. *Muscularis* was harvested as described in section 2.2.1.3 at indicated time points after LPS administration. Sphingolipids were extracted from *muscularis* layer and analysed by HPLC-MS/MS as described in section 2.2.4. **A, B)** Data are averages of triplicate measurements and expressed relative to control values obtained at t=0. Significantly different from control values (* $p < 0.05$, ** $p < 0.01$).

3.4.2. Involvement of sphingosine kinase in LPS induced IL-6 cytokine production in RISM cells

Our findings that intraperitoneal injection of LPS to rodents results in an increase of S1P and C1P levels only in the intestinal *tunica muscularis externa*, prompted us to analyse the potential involvement of these two sphingolipid-phosphates in LPS-induced inflammatory responses in primary cultured RISM cells. A recent study showed that sphingosine kinase (SK) is activated by LPS and mediates LPS-induced cell survival in macrophages [Wu et al., 2004]. However, studies that address whether or not SK is involved in LPS-mediated production of cytokines are not available.

We have therefore examined the effect of LPS on IL-6 production in primary cultured RISM cells. The cells were treated for 4 h with 0.1 and 1 $\mu\text{g/ml}$ LPS, respectively. Control cells were treated with the corresponding amount of vehicle. At the respective time point, the media were collected and subjected to ELISA assay to determine the concentration of IL-6 released in the medium. As shown in Fig. 24 A, S1P-induced IL-6 production in RISM cells increased significantly upon 1 $\mu\text{g/ml}$ LPS treatment (262% \pm 45 %). To determine the necessity of SK as a mediator of LPS-induced IL-6 elevation, cells were treated for 4 h with LPS (1 $\mu\text{g/ml}$) in the absence or presence of 10 μM N, N-dimethylsphingosine (DMS), a potent inhibitor of SK. IL-6 content was determined in the culture media as described in 2.2.8.2. We observed that LPS induced-IL-6 secretion in primary cultured RISM cells was suppressed by

DMS, suggesting that SK activation is required for expression of IL-6 (Fig. 24 A). In addition we measured the enzymatic activity of SK after treatment with LPS for 4 h. As shown in Fig. 24 B, LPS induced a clear increase of SK activity by about $230\% \pm 18\%$. Note the generation of C1P suggesting the possible activation of ceramide kinase (CK) by LPS (Fig. 24 B). It can, however, not be excluded that in these cells SK does not exhibit substrate specificity, thus phosphorylating both, sphingosine and ceramide following LPS treatment. Taken together these results suggest that LPS-induced IL-6 secretion is dependent of SK and might also involve CK.

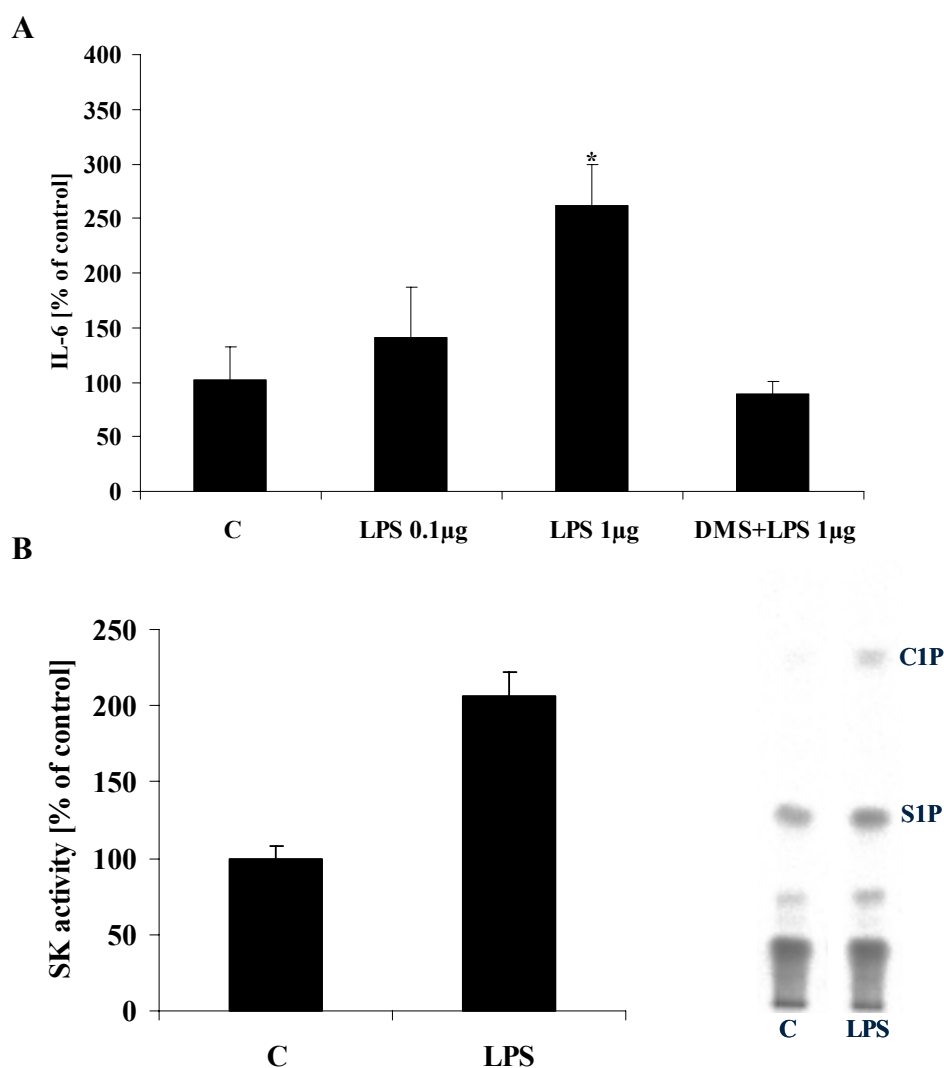


Fig. 24 Involvement of SK in LPS-induced IL-6 secretion in primary cultured RISM cells. **A)** Cells were incubated with the indicated concentrations of LPS in the absence or presence of DMS (10 μ M). Control cells (C) were always treated accordingly in the presence of vehicle. After 4 h media were collected and subject to ELISA assay as described in section 2.2.8.2. Data are means \pm S.D. from three independent experiments. * $p < 0.05$ treated with 1 μ g/ml LPS vs. control cells. **B).** Cells were incubated for 4 h with vehicle (C) or with 1 μ g/ml LPS. After 4 h cells were harvested and SK activity was measured in cell homogenates using selective buffer conditions as described in section 2.2.5. 32 P-labelled lipids were resolved by TLC using 1-butanol/methanol/acetic acid/water (80:20:10:20) as solvent system and visualized by autoradiography using the bio-imaging analyzer Fujix bas 1000 software, Tina 2.09. The radioactive spots corresponding to S1P and C1P were identified using S1P and C1P as standards. Data are means \pm SD from two independent experiments, each with double determinations.

4. Discussion

Gentle manipulation of the small intestine as well as LPS cause ileus by initiating an inflammatory cascade within the intestinal smooth muscle layer accompanied by decrease in smooth muscle contractility. Various mediators have been found to be involved in the inflammatory cascade. Since sphingolipids are emerging as a novel class of bioactive lipids in the regulation of inflammation, in this study we tried to find out whether sphingolipids could contribute to the onset of inflammatory responses induced by surgical manipulation of the small intestine or by endotoxemia.

4.1. Surgical manipulation of the bowel increases the contents of sphingosine-1-phosphate and ceramide-1-phosphate in the intestinal muscularis

To investigate the potential role of sphingolipids in postoperative ileus, we used an *in vivo* standardized surgical rodent model of intestinal manipulation. For an accurate analysis we did not use the entire small bowel but the selectively isolated *muscularis* and *mucosa* layers. Since ceramide was the primary sphingolipid found to play a role in inflammatory responses we started by analysing its content in intestinal *mucosa* and *muscularis* after surgical manipulation. Data obtained by TLC showed no significant changes in the content of ceramide at any time point after surgical manipulation versus control. Additionally, we did not find any difference in the amount of ceramide derivatives such as SM or sphingosine in both layers. Currently, technologies used for lipid analyses include besides chromatography like TLC [Touchstone, 1995; van Echten-Deckert, 2000], nuclear magnetic resonance (NMR) and mass spectrometry (MS). Although, NMR can be used to measure simultaneously all kinds of small molecule metabolites, it has the major disadvantage that it is relatively insensitive compared to MS-based technique. MS is both sensitive and can be very specific [van Echten-Deckert and Herget, 2006]. MS is used to identify and quantify metabolites after separation by gas chromatography (GC), high performance liquid chromatography (HPLC) [Muthing, 2000; Sullards and Merrill, 2001] or capillary electrophoresis. GC-MS offers high chromatographic resolution, but requires chemical derivatization for many biomolecules and only volatile chemicals can be analysed without derivatization. Therefore some large and polar metabolites cannot be analysed by GC. Instead, HPLC has lower chromatographic resolution but it has the advantage that a much wider range of lipids can be measured. For a precise quantification of sphingolipid content postsurgically, we employed HPLC-MS/MS

that also enabled determination of sphingolipids present in very low amounts in tissues. HPLC-MS/MS showed that the sphingolipid profiles in *mucosa* and *muscularis* are not identical. This is not surprising since they consist of different cell types and the inflammation mainly occurs in the muscularis layer. Whereas no significant changes of any of the sphingolipids analysed have been observed in the *mucosa* layer after intestinal manipulation, the *muscularis* exhibited a time dependent increase in the content of C1P and S1P. This is a remarkable finding indicating for the first time an implication of these two bioactive lipids in an inflammatory response in a pathological situation [Dragusin et al., 2006]. Moreover, these results show a direct correlation of the content of these bioactive sphingolipids in the intestinal *muscularis* and the inflammatory processes observed in this tissue layer postsurgery. We assume that surgical manipulation leads to an increase in the content of S1P and C1P by inducing the expression of sphingosine kinase (SK) and ceramide kinase (CK), respectively. SK, the enzyme that generates S1P, was localized in murine Peyer's patches [Baumruker et al., 2005], the lymphatic nodules that are found predominantly in ileum. Note that we indeed detected a significant increase in the activity of SK and also of CK after mechanical manipulation of primary cultured RISM cells. However, we cannot exclude that, cells other than RISM cells that occur in the *muscularis* layer contribute to an augmentation of S1P and C1P content. It can also be speculated that the increase in S1P derives from platelets. S1P is abundantly stored in platelets and released extracellularly upon stimulation [Yatomi et al., 1995; Yatomi et al., 1997]. Therefore, an increased level of S1P might occur under conditions in which the integrity of the vascular endothelial cells is disturbed. Note that, vascular endothelium is normally injured after trauma and shock [Lichtman, 2001].

Usually, tissues contain several sphingolipid subspecies, which differ in the degree of saturation and length of fatty acids as well as in their sphingoid base. The *muscularis* layer after intestinal manipulation exhibited a larger fraction of the C16:0 fatty acid C1P subspecies *versus* C18:0 when compared to non-manipulated samples. By contrast, C18 ceramide was found to be the prevailing subspecies in the intestinal *muscularis*. Furthermore, ceramide kinase was recently characterized regarding its substrate specificity in HEK293 cells and the enzyme demonstrated no preference for ceramides with saturated (C16, C18) versus unsaturated (C24:1) acyl chain lengths [Sugiura et al., 2002]. Thus, the finding that C16-C1P was the major subspecies of C1P found in *muscularis* after surgical manipulation could not be explained by substrate preference but rather by subcellular availability of the substrate. In this context, it was demonstrated that the ceramide transport protein (CERT) preferentially transported C16 ceramide from the ER to the *trans*-Golgi network where CK is localized

[Hanada et al., 2003; Kumagai et al., 2005]. We therefore, suggest that intestinal manipulation could affect the transport of C16 ceramide in RISM cells. Although the changes in C1P subspecies are subtle, they demonstrate that manipulation of the small intestine alters both the total amount and the structure of C1P.

4.1.1. Putative implication of sphingosine-1-phosphate and ceramide-1-phosphate in inflammatory responses initiated by surgical manipulation

It has been reported that surgical manipulation also increases the number of mast cells and induces their activation [Kalff et al., 1998]. Once activated, these cells degranulate and release potent inflammatory mediators such as histamine, TNF- α , proteases and produce newly synthesized mediators including prostaglandins and a variety of cytokines [Galli et al., 1991; Wershil, 1995]. Moreover, mast cells activation can amplify the recruitment of leukocytes through the release of histamine and the up-regulation of adhesion molecules [Kubes and Kanwar, 1994; Wershil et al., 1996]. A massive and persistent extravasation of leukocytes into the intestinal *muscularis* also occurs after surgical manipulation due to release of proinflammatory cytokines by the dense network of resident muscularis macrophages. Many studies pointed to a role of sphingolipids in mediating priming and activation of various cells of the immune system [El Alwani et al., 2006]. One could hypothesize that S1P and C1P participate in inflammatory processes that accompany postoperative ileus by inducing mast cell degranulation and/or leucocyte extravasation. For instance, it was found that increased S1P levels lead to activation of mast cells and the release of inflammatory mediators such as leukotrienes and cytokines [Prieschl et al., 1999]. Furthermore, it was observed that cross-linking of a high-affinity receptor for IgE (Fc ϵ RI) in mast cells activates SK1 leading to generation and secretion of S1P. This in turn activates its receptors, S1P₁ and S1P₂ that are required for mast cell degranulation and chemotaxis [Jolly et al., 2002]. S1P activates its receptors on the same and /or neighboring cells in an autocrine or paracrine manner. Up to now, it was a mystery how S1P produced inside cells could reach its receptors on the cell surface. Membrane lipids do not spontaneously exchange across lipid bilayers since the polar head groups do not easily transverse the hydrophobic interior of the membrane. Therefore, cells have many special transport proteins for this purpose. In a very recent study it was shown that S1P is transported out of rodent and human mast cells by ABCC1, an ATP-binding cassette (ATP) transporter [Mitra et al., 2006]. Additionally, it was reported that mice deficient in ABCC1 display impaired inflammatory responses due to decreased secretion of leukotriene, LTC₄ [Wijnholds et al., 1997]. One could speculate that the impaired

inflammatory responses might be partly caused by decreased secretion of S1P that acts not only in an autocrine manner to regulate mast cells functions [Olivera and Rivera, 2005] but also it activates other cells involved in inflammatory responses [Graeler and Goetzl, 2002; Jolly et al., 2002; Roviezzo et al., 2004]. CK and its product, C1P were also proposed to function in degranulation of mast cells [Mitsutake et al., 2004]. In addition, cell migration is also an important event in inflammatory processes, which is facilitated by the expression of adhesion proteins on endothelial cells. It was found that S1P acts as an effective regulator of migration of freshly isolated human neutrophils across endothelial cells [Kawa et al., 1997; MacKinnon et al., 2002]. In addition, it was shown that expression of adhesion molecules on endothelial cells in response to TNF- α is mediated by SK activation and S1P generation [Xia et al., 1998]. Likewise, S1P is a potent chemotactic factor for lymphocytes. It stimulates migration and enhances survival of splenic T cells, these actions being dependent on the differential expression of S1P receptors and S1P concentration in blood [Graeler and Goetzl, 2002; Rosen and Goetzl, 2005]. Moreover, S1P and C1P favour survival of macrophages by preventing ceramide accumulation, thus blocking apoptosis [Gomez-Munoz et al., 2003; Gomez-Munoz et al., 2005]. By contrast, ceramide and sphingosine are well known inducers of programmed cell death in mast cells [Itakura et al., 2002].

4.2. Sphingosine-1-phosphate and ceramide-1-phosphate induce inflammatory responses in RISM cells

It was shown before that intestinal manipulation induces COX-2 expression, which results in increased synthesis of prostaglandins within the *muscularis* [Schwarz et al., 2001]. Up-regulation of COX-2 could be attributed to resident macrophages but a contribution of smooth muscle cells or other cellular populations that inhabit the intestinal *muscularis externa* cannot be ruled out. Regardless of the complexity of the inflammatory response within the intestinal *muscularis*, probably involving several cell types, our study focused on elucidating the involvement of sphingolipids in inflammatory reactions using primary cultured RISM cells. The latter represent not only the prevailing cell type in the intestinal *muscularis* layer but have been shown to be mostly affected in their function, as suggested by well documented post-operative dysmotility [Kalff et al., 1998; Kalff et al., 1999b].

Up to now, most of the research concerning the involvement of sphingolipids in inflammatory processes focused on diverse cell lines, whereas less work addressed their role in primary cultured cells. First, we determined whether ceramide as precursor of S1P and C1P or neutral SMase that generates endogenous ceramide induced COX-2 expression in RISM cells. We

found that ceramide and SMase do not induce a significant increase in COX-2 protein expression in these cells. These results together with the finding that intestinal manipulation does not change the content of ceramide in intestinal *muscularis* exclude the possible involvement of ceramide in inflammatory processes that accompany postoperative ileus. Initial reports regarding a role of sphingolipids in inflammation showed that sphingosine and a cell-permeable ceramide (C2-ceramide) enhanced IL-1-mediated PGE₂ formation by COX activation in human fibroblasts [Ballou et al., 1990; Ballou et al., 1992]. Furthermore, treatment of human mammary epithelial cells with ceramide or SMase was shown to enhance COX-2 expression and PGE₂ formation [Subbaramaiah et al., 1998]. However, the hypothesis that ceramide is involved in COX-2 induction does not reconcile easily with the proposed role of ceramide in inducing cell death and the proinflammatory nature of COX-2 in cancer [Pettus et al., 2004b]. On the other hand, ceramide was shown to inhibit COX-2 induction in response to LPS by inhibition of NF- κ B and AP-1 [Hsu et al., 2001].

Therefore, we investigated the potential involvement of S1P and C1P in inflammatory processes in RISM cells, the sphingolipid-phosphates whose levels specifically increased in the *muscularis* layer after intestinal manipulation.

4.2.1. Sphingosine-1-phosphate stimulates cyclooxygenase-2 induction and PGE₂ production in RISM cells

There is experimental evidence for the involvement of S1P in COX-2 regulation. Our data suggest that exogenous S1P is sufficient to induce COX-2 and PGE₂ production in a time and concentration dependent manner in primary cultured RISM cells [Dragusin et al., 2006]. Our results are consistent with findings of others, closely correlating S1P with the metabolism of eicosanoids [Pettus et al., 2005]. It was therefore not surprising that S1P modulates PGE₂ synthesis. But it was of some surprise that modulation of PGE₂ synthesis by S1P, previously described primarily in diverse cell lines [Pettus et al., 2003b; Chalfant and Spiegel, 2005] is also valid in primary cultured RISM cells. In a recent study, the effects of ceramide, sphingosine and S1P on COX-2 induction were compared under identical conditions and the results showed that the actions of S1P were the most potent. In addition, the actions of ceramide and sphingosine were prevented when SK1 activity was knocked down via RNAi, thus demonstrating that the actions of these two sphingolipid metabolites require metabolic conversion to S1P [Pettus et al., 2003b]. Moreover, RNAi directed against S1P lyase and S1P phosphatase, which should increase S1P levels, augmented COX-2 activation and consequently PGE₂ generation. A critical role of SK1 in mediating PGE₂ production was also

observed in A 549 cells [Billich et al., 2005]. A recent study suggested moreover that SK1 expression is upregulated in rat colon tumors implying an involvement of SK1/S1P pathway in the regulation of the COX-2/PGE₂ pathway in colon cancer cells [Kawamori et al., 2005]. Taken together, these data define a role of S1P in COX-2 induction and PGE₂ production.

4.2.2. Sphingosine-1-phosphate-induced cyclooxygenase-2 expression is pertussis toxin sensitive and mediated by MAPKs in RISM cells

It is well known that S1P can act either as an extracellular ligand by binding to a family of G-protein coupled receptors (GPCRs) or as an intracellular second messenger with so far unknown direct targets [Spiegel and Milstien, 2003]. All five members of the S1P-receptor family can be coupled to G i/o-proteins [Ishii et al., 2004]. Since the latter are inactivated by pertussis toxin (PTX) via ADP-ribosylation, we used this toxin to find out in a first approach whether the stimulatory effect of S1P on COX-2 expression is receptor-mediated. Previous studies on human airway smooth muscle cells showed that modulation of cytokines by S1P was PTX independent indicating that it did not involve G-protein coupled receptors. By contrast, in RISM cells the PTX-sensitivity of S1P induced COX-2 expression implicates the involvement of Gi/o coupled S1P-receptors. Interestingly it was found that S1P₂ plays a key role in myogenic differentiation [Donati et al., 2005]. Based on several previous studies [Ishii et al., 2004] we assume that S1P₂ also mediates S1P-induced COX-2 expression in RISM cells. Note that Broere R. from our group identified the presence of S1P₂ in RISM cells by RT-PCR.

S1P-receptor-mediated effects often imply MAPK signalling pathways. ERK MAPK pathway usually promotes cell proliferation whereas activation of SAPK/JNK is important in controlling apoptosis and p38 MAPKs appear to be key regulators of inflammatory response in immune cells [Johnson and Lapadat, 2002]. Due to its tumor-promoting function, inflammation is often considered a proliferative rather than an apoptotic process [Chalfant and Spiegel, 2005]. Thus, one would imply activation of the ERK and/or p38-signalling pathway upstream of COX-2 expression. In primary cultured RISM cells, S1P-induced COX-2 expression appears to involve phosphorylation of both, ERK and p38 MAPK signalling pathways. This was proved by using two different specific inhibitors, PD-98059 and SB 239063, for ERK and for p38 MAPK inhibitors, respectively. In human amnion-derived WISH cells [Kim et al., 2003] and also in human coronary artery smooth muscle cells [Daminin et al., 2005], S1P-modulated COX-2 expression occurs only by activation of ERK signalling pathway. Remarkably, our results are similar with those obtained for the pro-

myogenic and anti-proliferative S1P₂-mediated action of S1P in myoblasts [Donati et al., 2005]. Thus, it appears that S1P induces two rather opposite physiological processes, namely morphogenesis and inflammation, albeit in undifferentiated myoblasts and differentiated smooth muscle cells, respectively, using similar signalling pathways. This finding once more emphasises that similar signalling pathways can induce different cellular responses depending on the developmental stage of a cell.

4.2.3. Sphingolipid kinases mediate IL-6 cytokine production in response to TNF- α in RISM cells

IL-6 is a prototypical inflammatory cytokine whose expression was induced in the *muscularis* layer through the intestinal manipulation procedure [Wehner et al., 2005].

In the present study, the effect of S1P on cytokine production was also addressed. Our data suggest that exogenous S1P induces a time and concentration dependent increase of IL-6 secretion in a PTX-sensitive manner. Interestingly, in human airway smooth muscle cells IL-6 secretion by S1P was PTX-insensitive [Ammit et al., 2001] reflecting either activation of (non-Gi-coupled) receptors or intracellular actions of internalized S1P. Moreover, the direct measurement of IL-6 release after manipulation indicated that extravasated leukocytes are major producers of IL-6 [Wehner et al., 2005]. However, previous studies showed that intestinal smooth muscle cells have the capacity to produce IL-6 in response to IL-1 β or TNF- α [Khan et al., 1995; Ng et al., 2003]. In the present study, we found that the induction of IL-6 secretion by TNF- α was suppressed by a specific SK inhibitor, dimethylsphingosine (DMS), a pharmacological inhibitor of both SK1 and SK2 suggesting that TNF- α acts through induction of SK in RISM cells. Indeed, we found an increase in SK activity upon stimulation of RISM cells with TNF- α . Interestingly, in TNF- α stimulated RISM cells we also observed the generation of C1P indicating that either in these cells SK does not exhibit substrate specificity thus phosphorylating both, sphingosine and ceramide or that a simultaneous activation of CK occurs. Since ceramide content is quite elevated in these cells, it appears more likely that the activity of CK was monitored simultaneously. The involvement of SK in TNF- α induced cytokine production was also reported in A549 carcinoma cells. Studies with RNAi against SK1 and SK2 reported that SK1 but not SK2 is activated in response to TNF- α and IL-1 β [Billich et al., 2005]. This is not surprising since SK1 has been implicated in the stimulation of cell growth and inflammatory responses, promotion of cell migration and inhibition of apoptosis. By contrast, SK2 has been involved in the inhibition of cell growth and stimulation of apoptosis [Le Stunff et al., 2004].

The question arises on how SK1/and or its reaction product S1P might induce cytokine production. Xia et al. using HEK 293T cells as a model system showed that SK1 directly interacts with TNF receptor-associated factor 2 (TRAF2), resulting in activation of the enzyme, which in turn is required for TRAF2-mediated activation of NF- κ B [Xia et al., 2002]. Moreover, the dependence of proinflammatory mediator production on SK1 might be explained by interaction of S1P generated intracellularly with a yet unknown intracellular receptor or with extracellular receptors following secretion of S1P.

4.2.4. Ceramide-1-phosphate is a potent and specific inducer of arachidonic acid release and PGE₂ formation in RISM cells

The finding that surgical manipulation induces an increase in the content of C1P in intestinal *muscularis*, determined us to investigate its possible implication in inflammatory responses in primary cultured RISM cells. We found that unlike S1P, C1P had no effect on COX-2 protein expression but like S1P also triggered PGE₂ production. Therefore, we assumed that C1P acts on the initial rate-limiting step involved in PGE₂ synthesis, namely PLA₂ that catalyses the release of arachidonic acid from phospholipids. Our data indicate that C1P is an activator of PLA₂ by inducing a time-dependent increase in arachidonic acid release. In addition, treatment of RISM cells with related lipids did not mimic C1P effects on arachidonic acid release, thus supporting the specificity of C1P in this regard [Dragusin et al., 2006]. Our findings are consistent with the ones from another study in which treatment of A549 cells with C1P alone stimulated arachidonic acid release and PGE₂ production [Pettus et al., 2003b]. The role of sphingolipids in cPLA₂ regulation has come from an unexpected source. It is known that many types of venom induce acute inflammation by the activity of soluble venom PLA₂. By contrast, it was reported that unlike SMase C that hydrolyses SM to ceramide, SMase D from *Loxosceles recluses* venom generates C1P [Merchant et al., 1998]. This finding led to the assumption that many of the actions attributed to ceramide might be explained by the action of C1P. It was reported that treatment of cells with SMase D elicited arachidonic acid release, whereas SMase C, which generates ceramide had no effect. Moreover, IL-1 β and calcium ionophore A23187 produced an increase in CK activity and elevated C1P levels. More important, RNAi targeted against CK, inhibited arachidonic acid release and PGE₂ synthesis in response to IL-1 β and A23187 implicating CK as a mediator of cPLA₂ activation [Pettus et al., 2003a]. Furthermore, it was demonstrated that C1P enhanced cPLA₂ association with the membrane and was able to induce translocation of cPLA₂ to the

Golgi and perinuclear regions. Collectively, these results implicate CK/C1P in activation of cPLA₂ upstream of COX-2 induction.

4.2.5. Mechanical stress activates sphingolipid kinases in RISM cells

To find out whether mechanical stress assumed to be causative for the inflammatory processes of intestinal *muscularis* is correlated with increased levels of S1P and C1P, muscle cells were subjected to mechanical stretch to mimic the insult that has been reported to induce postoperative ileus. We found that mechanical stress indeed activates SK, and simultaneously an enhancement of C1P content was observed. The latter observation could be explained by activation of CK during mechanical stress, its substrate, ceramide being highly abundant in RISM cells. These results together with the ones obtained after manipulation of small intestine suggest that activation of SK and CK occur quite early after manipulation (between 4 h - 6 h). Moreover, in RISM cells, mechanical stretch induces release of inflammatory mediators suggesting that together with other cells that reside within the *muscularis* layer they participate in inflammatory responses during postoperative ileus.

In conclusion, we could show for the first time that S1P and C1P are potential proinflammatory mediators using manipulated rat small intestine and RISM cells. It has been suggested very recently that C1P and S1P may act in concert to regulate PGE₂ production [Pettus et al., 2005]. This suggestion is strongly supported by the following findings of the present study: i) the similarity of the time course concerning the increased levels of both sphingolipid metabolites in the muscle layer after intestinal manipulation, ii) stimulation of COX-2 expression and arachidonic acid release by S1P and C1P, respectively and iii) the early but transient versus a continuously increase of PGE₂ formation induced by S1P and C1P, respectively (Fig. 25). The latter finding also supports the hypothesis that S1P primes the system for PGE₂ synthesis by inducing COX-2 [Chalfant and Spiegel, 2005]. The fact that both stimulate synthesis of PGE₂ and that S1P induces IL-6 release makes them interesting targets for development of novel anti-inflammatory drugs.

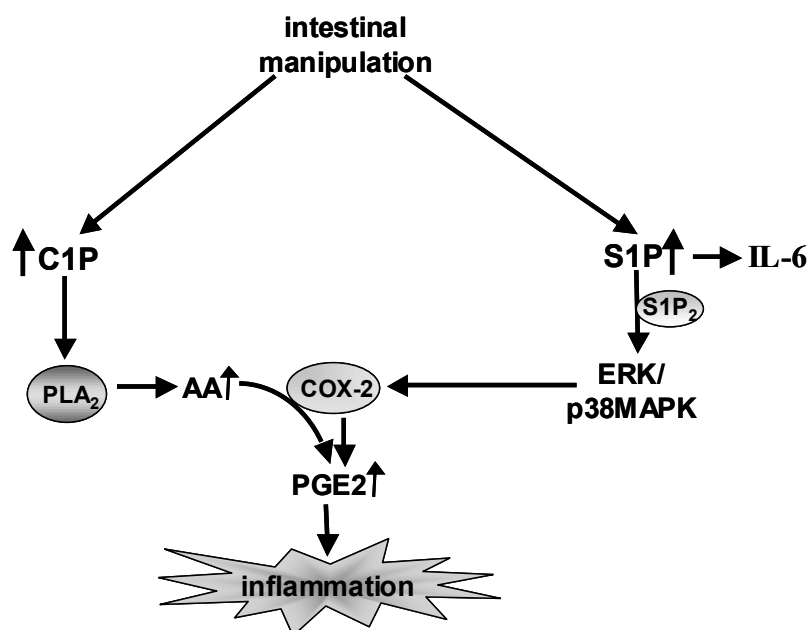


Fig. 25 Scheme of proposed mechanism of sphingolipid involvement in inflammatory responses generated by surgical manipulation of the bowel.

4.3. Endotoxemia increases the contents of sphingosine-1-phosphate and ceramide-1-phosphate in the intestinal muscularis

The intestine has been hypothesized to play an important role in both septic shock and multiple-organ failure as an endogenous source of not only bacterial products but also proinflammatory cytokines, which circulate systemically leading to functional detriment of distant organs [Mainous et al., 1995; Grotz et al., 1999]. However the intestine itself is a major target of self-derived bacterial products and cytokines. It was shown that intraperitoneal injection of endotoxin (LPS) elicits an inflammatory response within the intestinal *muscularis* by activation of resident macrophages. LPS-activated macrophages were able to secrete a plethora of cytokines and kinetic active substances (NO, PGE₂) resulting in recruitment of numerous leukocytes [Sweet and Hume, 1996]. This inflammatory response in the intestinal *muscularis* was found to suppress smooth muscle contractility, LPS being known as a causative factor of sepsis-induced ileus [Eskandari et al., 1997; Eskandari et al., 1999]. In order to study the possible involvement of sphingolipids in LPS-induced inflammatory reactions, we used a well-established sepsis model that implies bolus intraperitoneal administration of LPS. Lipid content was determined in both, *mucosa* and *muscularis* at different time intervals after LPS administration. In the *mucosa* layer no significant changes in any of the sphingolipid analysed could be detected. By contrast, in tunica *muscularis* we found an increase in the content of S1P and C1P already after 3 h, the maximum being

reached at 12 h post LPS injection. It has been previously shown that LPS initiates an inflammatory response that consists of an activation of a normally quiescent network of resident intestinal muscularis macrophages. Moreover, LPS induced recruitment of polymorphonucleated leucocytes within intestinal *muscularis* maximum being reached at 12 h after LPS administration [Buchholz, 2005]. Recruited cells degranulate and release inflammatory mediators including reactive oxygen intermediates, nitric oxide, prostaglandins and histamine, which may suppress smooth muscle contractile activity [Eskandari et al., 1997]. It was found that S1P acts as an effective regulator of migration of freshly isolated human neutrophils across endothelial cells [Kawa et al., 1997; MacKinnon et al., 2002]. Therefore, one might suggest the involvement of S1P and C1P in recruitment of leucocytes in the intestinal *muscularis* explaining their increased levels 12 h after LPS injection. Leucocyte infiltration in rat intestinal *muscularis* was also reported at 24 h after gentle surgical intestinal manipulation. Leucocyte extravasation occurs as a result of a significant up-regulation of adhesion molecules within the vasculature of rat intestinal *muscularis* [Kalff et al., 2003]. Similar results regarding activation of resident intestinal muscularis macrophages, induction and release of inflammatory cytokines (IL-6, IL-1 β , TNF- α) and kinetic active mediators (NO, PGE₂) as well as recruitment of leukocytes could be shown in human small bowel specimens harvested during abdominal procedures [Kalff et al., 2003].

4.4. Sphingolipid kinases mediate LPS-induced inflammatory responses

Our results obtained with intestinal tissue after LPS injections indicate that S1P and or C1P might mediate inflammatory responses. Even though it was suggested that resident muscularis macrophages release various inflammatory cytokines after LPS injection, we cannot exclude that smooth muscle cells also participate in the inflammatory cascade. In the present study we found that RISM cells have the capacity to produce IL-6 in response to LPS. Moreover, this process is mediated by S1P formation since IL-6 secretion was suppressed by DMS, a specific SK inhibitor. In addition, the enzymatic activity of SK and increased after treatment of RISM cells with LPS suggesting that SK mediate LPS-induced IL-6 production. We have also observed an increase formation of C1P indicating the possible activation of CK. Taken together our data suggest that not only resident muscularis macrophages but also RISM cells might participate in endotoxemia involving sphingolipid phosphorylation. A recent study showed that LPS stimulates SK activity in a murine macrophage cell line and that SK is involved in LPS activation of NF- κ B [Wu et al., 2004]. The transcription factor NF- κ B is

involved in the expression of many inflammatory mediators [Karin and Ben-Neriah, 2000]. It would be interesting to investigate whether LPS also activates SK in *muscularis* macrophages. Our findings after LPS administration fit together with the ones obtained upon intestinal manipulation and mechanical stretch of RISM cells indicating the involvement of S1P and C1P in inflammatory responses derived thereof. This is not surprising since both sepsis- and postoperative-induced ileus share similar mechanisms.

5. References

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Erklärung

Hierhin versichere ich, die vorliegende Arbeit selbständig und unter ausschließlicher Verwendung der angegebenen Hilfsmittel verfasst zu haben, wobei ich die von mir verwendeten Quellen als solche gekennzeichnet habe.

Bonn, December 2006

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