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THEMED SECTION: MEDIATORS AND RECEPTORS IN THE RESOLUTION OF INFLAMMATION

REVIEW

Sphingolipids in inflammation: pathological implications and potential therapeutic targets

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Sphingolipids are formed via the metabolism of sphingomyelin, a constituent of the plasma membrane, or by *de novo* synthesis. Enzymatic pathways result in the formation of several different lipid mediators, which are known to have important roles in many cellular processes, including proliferation, apoptosis and migration. Several studies now suggest that these sphingolipid mediators, including ceramide, ceramide 1-phosphate and sphingosine 1-phosphate (S1P), are likely to have an integral role in inflammation. This can involve, for example, activation of pro-inflammatory transcription factors in different cell types and induction of cyclooxygenase-2, leading to production of pro-inflammatory prostaglandins. The mode of action of each sphingolipid is different. Increased ceramide production leads to the formation of ceramide-rich areas of the membrane, which may assemble signalling complexes, whereas S1P acts via high-affinity G-protein-coupled S1P receptors on the plasma membrane. Recent studies have demonstrated that *in vitro* effects of sphingolipids on inflammation can translate into *in vivo* models. This review will highlight the areas of research where sphingolipids are involved in inflammation and the mechanisms of action of each mediator. In addition, the therapeutic potential of drugs that alter sphingolipid actions will be examined with reference to disease states, such as asthma and inflammatory bowel disease, which involve important inflammatory components. A significant body of research now indicates that sphingolipids are intimately involved in the inflammatory process and recent studies have demonstrated that these lipids, together with associated enzymes and receptors, can provide effective drug targets for the treatment of pathological inflammation.
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Keywords: inflammation; sphingolipids; sphingosine 1-phosphate; ceramide; cyclooxygenase; cytokine; lymphocytes; asthma; colitis

Abbreviations: c/EBP, CCAAT/enhancer binding proteins; C1P, ceramide 1-phosphate; COX-2, cyclooxygenase-2; $cPLA_2$, cytosolic phospholipase A_2; HDL, high-density lipoproteins; IBD, inflammatory bowel disease; IL, interleukin; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; PGE_2, prostaglandin E_2; SK, sphingosine kinase; SMase, sphingomyelinase; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; TNF, tumour necrosis factor-α

Introduction

It is now clear that the membrane cycling of sphingomyelin, involving its degradation and re-synthesis via a number of intermediate steps, is not merely concerned with maintenance of membrane integrity (Kolesnick, 1987; Okazaki *et al.*, 1989). Many of the lipid intermediates formed in what is termed 'the sphingomyelin cycle' are mediators of cellular functions in their own right (Kolesnick, 1991; Hannun and Bell, 1993; Spiegel and Milstein, 1995). Altered levels of these different sphingolipid species can have profound consequences on cell phenotype and indeed the balance of interdependent sphingolipids produced in the cell membrane can predict cellular behaviour (Cuvillier *et al.*, 1996). Emerging experimental evidence now points to an important role for sphingolipids in inflammation (Chalfant and Spiegel, 2005; El

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Alwani *et al.*, 2006). This involvement varies dependent upon the cellular context and the sphingolipid mediator involved. As these roles become clearer, new therapeutic targets are being identified providing scope for pharmacological intervention. This review will focus on the involvement of sphingolipid mediators in the inflammatory process and highlight exciting directions for novel therapeutic agents.

Sphingomyelin cycle

The breakdown of sphingomyelin is regulated by the activity of sphingomyelinase (SMase) enzymes (Goni and Alonso, 2002; Marchesini and Hannun, 2004) (Figure 1). SMases are activated by a variety of stimuli, including inflammatory cytokines, growth factors, G-protein-coupled receptors and cell stress. There are several different isoforms of SMase in mammalian cells: acid SMase (optimum activity at around pH 5), neutral (Mg^{2+}-dependent) SMase and secretory SMase (Samet and Barenholz, 1999). The role of each of these isoforms is not entirely clear but likely to depend on a number of factors, such as intracellular localization and mechanisms of activation. Acid SMase is found mostly in lysosomes (where an acid environment occurs) (Fowler, 1969), whereas secretory SMase (derived from the acid SMase gene) is targeted to the Golgi secretory pathway (Schissel *et al.*, 1996) and could potentially restrict the effects of sphingolipid mediators produced in certain intracellular locations. Neutral SMase is a membrane-bound protein and appears to be ubiquitously expressed in mammalian cells (Chatterjee, 1999). Activation of SMase isoforms leads to the breakdown of sphingomyelin to ceramide (Figure 1), which can be either phosphorylated by ceramide kinase to form C1P or degraded further by ceramidase to produce sphingosine (Hannun and Bell, 1993). Similarly, sphingosine can be phosphorylated by sphingosine kinases to sphingosine 1-phosphate (S1P). Two isoforms of sphingosine kinase (SK) are expressed in mammalian cells, SK1 and SK2 (Olivera *et al.*, 1998; Liu *et al.*, 2000a) and, although both can phosphorylate sphingosine, there is some divergence of function (discussed later) (Alemany *et al.*, 2007). The metabolism of S1P can occur via two routes, either by dephosphorylation (through the actions of sphingosine

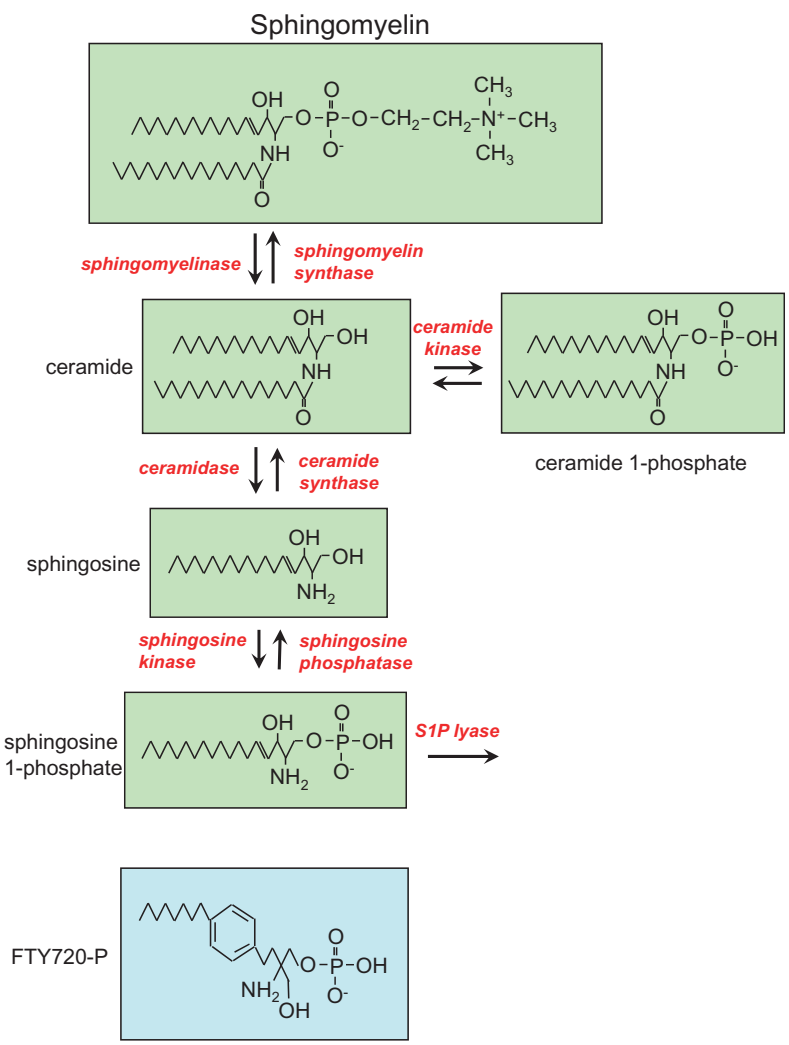

Figure 1 The sphingomyelin cycle. The structure of key sphingolipids and the major metabolic cycle is shown in schematic form. Enzymes for each step are highlighted in red. The structure of FTY720-P is shown for comparison.

phosphatase) or by S1P lyase resulting in degradation and removal from the sphingomyelin cycle (Pyne *et al.*, 2004; Kihara *et al.*, 2007). Sphingomyelin can be re-synthesized from sphingosine by the enzymes ceramide synthase (converting sphingosine to ceramide) and sphingomyelin synthase (ceramide to sphingomyelin). Another sphingolipid closely related to S1P, sphingosylphosphorylcholine (SPC), is also probably derived directly from sphingomyelin but via the action of another enzyme, sphingomyelin deacylase (Nixon *et al.*, 2008).

All sphingolipids have a common sphingoid backbone structure and have at least some actions that affect cell function. However, the lipids that have been most studied to date and therefore have greatest relevance to the regulation of cellular function are ceramide, C1P and S1P (Spiegel and Milstein, 1995). Indeed, because the formation and degradation of these sphingolipids are interconnected and interdependent, the increase of one lipid resulting in a concomitant decrease in another could be an important regulator for cell function. This is demonstrated by the sphingolipid 'rheostat' that regulates pro- and anti-apoptotic signals (Cuvillier *et al.*, 1996). An increase in ceramide with a concomitant decrease in S1P leads to activation of cell death pathways, whereas a decrease in ceramide and parallel increase in S1P results in stimulation of anti-apoptotic pathways. It should be noted therefore that the effects ascribed to one sphingolipid, while valid, may also involve reciprocal changes to other lipids.

Despite being related sphingolipids, ceramide, C1P and S1P have distinct modes of action and also occurrence in different cell types. While the occurrence and mode of action of S1P have been better described, those of ceramide and C1P remain less clear.

Ceramide

Ceramide can occur in cells with a variety of carbon chain lengths. The longer-chain-length C16-C24 fatty acids are most common physiologically, although shorter-chain ceramides are generally used in research (Sot *et al.*, 2005). Ceramide can be formed by the actions of SMases in the membrane (as mentioned previously) or formed by alternative routes, such as *de novo* pathways via ceramide synthase or the breakdown of glycosphingolipids (Ruvolo, 2003). In the case of synthesis via SMase, the site of SMase activity probably restricts the site of ceramide action. In addition, ceramides are very hydrophobic and can therefore only be located in cell membranes. Several signalling pathways can be regulated by ceramide, including a diverse range of protein kinases and phosphatases (Ruvolo, 2003). However, the exact nature of the interaction with such signalling proteins or other signalling components is still not entirely clear. Experimental evidence suggests that more than one mechanism may be involved. One of these is the formation of ceramide-rich domains in the plasma membrane (Huang *et al.*, 1996). These domains, similar to cholesterol-rich lipid raft domains, may act to assemble signalling complexes (Kolesnick *et al.*, 2000). Ceramide itself may promote protein–protein interaction at these sites, for example, dimerization of the TrkA receptor (MacPhee and Barker, 1999). Another potential mode of action through which ceramide mediates intracellular signalling is possibly via direct interaction with proteins that have a ceramide binding domain, for example, protein kinase C isoforms (Zhang *et al.*, 1997).

Ceramide 1-phosphate

Ceramide kinase has been localized to the cell cytoplasm or perinuclear regions (possibly dependent on cell type) and C1P may therefore occur in various intracellular locations. Ceramide kinase has a pleckstrin homology domain (which interacts with phosphatidylinositol 4,5-bisphosphate) important for enzyme activity (Kim *et al.*, 2005a) and a calmodulin binding motif (Mitsutake and Igarashi, 2005). The activity of this enzyme is increased by elevated $[Ca^{2+}]_i$. No plasma membrane receptor for C1P has been identified and its intracellular production suggests that it may interact with other signalling components. C1P has been shown to interact directly with signalling proteins, such as cytosolic phospholipase A_2 ($cPLA_2$) (Pettus *et al.*, 2004), an enzyme associated with inflammation through the production of arachidonic acid, the initial rate-limiting step in the production of inflammatory prostaglandins and leukotrienes.

Sphingosine 1-phosphate

S1P is to date the best described of the sphingolipid mediators. S1P produced by SK isoenzymes is up-regulated in cells via activation of G-protein-coupled receptors, growth factor receptors and cytokine receptors (Alemany *et al.*, 2007). SK localization in cells is dependent upon the isoenzyme examined and may be cytoplasmic or plasma membrane-bound (Venkataraman *et al.*, 2006). Translocation and plasma membrane targeting following activation have been observed in some cell types dependent upon stimulus (Johnson *et al.*, 2002). This suggests that S1P can potentially be produced at different intracellular locations varying with the SK isoenzymes activated/expressed, species or cell type examined. However, unlike ceramide and C1P, S1P also occurs naturally in plasma at relatively high concentrations (Yatomi, 2008). S1P is known to be an integral and functionally important constituent of lipoproteins (Murata *et al.*, 2000a), along with the related sphingolipid, SPC (Sachinidis *et al.*, 1999). The S1P concentration in plasma is around 200 nM and more than 60% is bound to lipoproteins (Okajima, 2002), with the majority bound to high-density lipoproteins (HDL) (86%). Proportionately less is bound to low-density lipoprotein (LDL) while oxidation of LDL reduces further the sphingolipid component. It is likely that the S1P component is involved in some of the cytoprotective effects of HDL (Kimura *et al.*, 2001; Nofer *et al.*, 2004). In addition to lipoproteins, there is another source of S1P that does not rely on *de novo* synthesis. S1P is found in abundance in platelets (Yatomi *et al.*, 1995), predominantly due to a very low expression of S1P lyase leading to accumulation of S1P (Yatomi *et al.*, 1997). Although it is not clear how S1P is stored in platelets, it is released rapidly by a carrier-mediated process following platelet activation (Kobayashi *et al.*, 2006). This release results in S1P serum concentrations estimated at up to 900 nM (Murata *et al.*, 2000b). S1P is therefore released at sites of platelet activation and various cell types are potentially exposed to

this sphingolipid, such as blood-borne cells (red blood cells, inflammatory cells), endothelial cells and vascular smooth muscle cells.

A major advance in understanding the physiological and pathophysiological role of S1P was the cloning and characterization of plasma membrane receptors with a high affinity for S1P (Pyne and Pyne, 2000; Sanchez and Hla, 2004). These receptors (initially termed EDG receptors, products of the endothelial differentiation gene) (Hla and Maciag, 1990; Lee *et al.*, 1998) are members of the seven-transmembrane, G-protein-coupled superfamily. To date, five subtypes of the S1P receptor ($S1P_{1-5}$) have been cloned. $S1P_{1-3}$ are expressed in many cell types with $S1P_4$ and $S1P_5$ restricted to specific cell types (recently reviewed, Meyer zu Heringdorf & Jakobs, 2007). These receptors can couple to multiple heterotrimeric G-proteins (with the exception of $S1P_1$ that couples only to $G_{\alpha}i$) and therefore have the potential to activate multiple signalling cascades (Windh *et al.*, 1999). $S1P_2$ and $S1P_3$ couple preferentially to $G_{\alpha}q$ (leading to activation of phospholipase C and intracellular Ca^{2+} release) and $G_{\alpha}12/13$ (activating the monomeric G-protein RhoA) (Sanchez and Hla, 2004). In various cell types agonist binding to $S1P_1$ receptors has been shown to activate mitogen-activated protein kinases (MAPK) (Okamoto *et al.*, 1998). In transgenic knockout mice, deletion of $S1P_2$ or $S1P_3$ do not reveal major changes in phenotype, while gene deletion of $S1P_1$ is embryonic lethal due to a failure of proper blood vessel formation (Liu *et al.*, 2000b). Through these receptors and associated signalling cascades, S1P has many different functional effects in varying cell types. Those relating to inflammation are further explored below.

Sphingolipids in inflammation

It is now becoming increasingly apparent that sphingolipids can be intimately involved in inflammation (Chalfant and Spiegel, 2005; El Alwani *et al.*, 2006). Many studies have demonstrated that, in some cell types, sphingolipids can have specific effects that are integral to regulation of the inflammatory response. Sphingolipids themselves may, in certain circumstances, initiate parts of the inflammatory process. As the modes of action of each of the sphingolipids described above are different, it is not surprising that the effects these lipids have on inflammation can occur via several different mechanisms (summarized in schematic form in Figure 2). Each sphingolipid and the specific effects on inflammation are discussed separately.

Ceramide

The first indications that ceramide produced by SMase turnover could play a role in inflammation were reported from studies examining the intracellular effects of the pleiotropic inflammatory cytokine, tumour necrosis factor-α (TNF) (Kim *et al.*, 1991; Mathias *et al.*, 1991). Of particular relevance to inflammatory responses, TNF can activate acid SMase resulting in ceramide production and subsequent activation of the pro-inflammatory transcription factor, nuclear factor-κB (NF-κB) (Schütze *et al.*, 1992). NF-κB are a family of transcription factors ubiquitously expressed in mammalian cells and

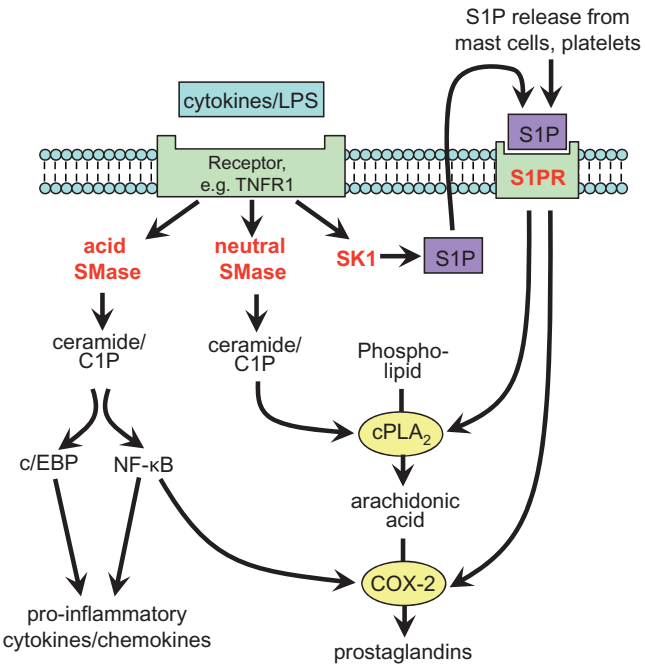

Figure 2 Main intracellular pathways showing the potential involvement of sphingolipids in inflammation. Possible sphingolipid therapeutic targets are shown in red. C1P, ceramide 1-phosphate; c/EBP, CCAAT/enhancer binding proteins; COX-2, cyclooxygenase-2; $cPLA_2$, cytosolic phospholipase A_2; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; S1P, sphingosine 1-phosphate; SK, sphingosine kinase; SMase, sphingomyelinase; TNF, tumour necrosis factor-α.

induce more than 150 different genes. Many of these genes encode cytokines and chemokines, such as interleukin-1β (IL-1β), IL-6, IL-8 and monocyte chemoattractant protein-1 in addition to pro-inflammatory enzymes, such as COX-2, all of which have important roles in inflammation (Xiao and Ghosh, 2005). COX-2 leads to the production of eicosanoids, including pro-inflammatory prostaglandin E_2 (PGE_2). Interestingly, TNF can activate neutral and acid SMases, but only activation of acid SMase results in NF-κB activation (Wiegmann *et al.*, 1994). Ceramide generated by TNF-induced activation of neutral SMase leads to increased activity of $cPLA_2$ (however, subsequent reports suggest that $cPLA_2$ activation may be via C1P, produced by the action of ceramide kinase, see below). Although these ceramide-dependent effects were observed in some cell lines, such as HL-60, several other studies have subsequently shown that the activation of SMases in a variety of different cell types is not essential for TNF-induced NF-κB activation. This included fibroblasts, endothelial cells and macrophages (Kuno *et al.*, 1994; Slowik *et al.*, 1996; Manthey and Schuchman, 1998). While SMase activity may not be required for this TNF-induced effect, these studies have demonstrated that ceramide itself can activate inflammatory pathways via NF-κB gene transcription. Ceramide can also up-regulate another family of transcription factors closely associated with inflammation. CCAAT/enhancer binding proteins (c/EBP) induce gene expression of several inflammatory proteins, including TNF, IL-6, IL-8 and IL-1β (Poli, 1998). In hepatocytes and macrophages, addition of ceramide activates c/EBP and also potentiates inflammatory effects induced by lipopolysaccharide (LPS) (Giltiay *et al.*, 2005; Cho *et al.*, 2003).

If indeed ceramide can play an important role in an inflammatory response, an effect on the induction of COX-2 and subsequent eicosanoid production might be expected. Several studies have demonstrated that ceramide can induce COX-2 expression and increase enzyme activity. In a human mammary epithelial cell line addition of short chain ceramides or neutral SMase induced COX-2 expression and led to an enhanced synthesis of PGE_2. This was via activation of several MAPK isoforms (Subbaramaiah *et al.*, 1998). Ceramide also induced expression of COX-2 and subsequently PGE_2 in epithelial-derived lung adenocarcinoma A549 cells (Newton *et al.*, 2001). While this was NF-κB-independent in A549 cells, ceramide-induced COX-2 up-regulation in macrophages required activation of NF-κB (Wu *et al.*, 2003).

Effects attributed to ceramide can possibly be the result of further conversion to C1P (see below) or even due to degradation to S1P. This can occur via the action of ceramidases. In L929 fibroblasts, TNF-induced induction of COX-2 and PGE_2 production were dependent on the activation of acid ceramidase and the resulting production of S1P (Zeidan *et al.*, 2006). Therefore, in some cases ceramide effects reported may be due to S1P and subsequent S1P receptor activation.

Although it is not clear if ceramide can regulate the inflammatory process in all cell types, it does indicate potential for an *in vivo* role. A recently published study has demonstrated that ceramide may mediate lung inflammation in cystic fibrosis (Teichgräber *et al.*, 2008). In a transgenic mouse model of cystic fibrosis, which was deficient in the cystic fibrosis transmembrane conductance regulator, an alkalinisation of intracellular vesicles in the respiratory tract led to a change in relative balance of acid SMase and ceramidase activity. This resulted in an accumulation of membrane ceramide and was responsible for subsequent pulmonary inflammation. Such studies demonstrate the pathophysiological importance of ceramide in inflammation and indicate a potential novel drug target in cystic fibrosis, which has yet to be fully investigated.

Ceramide 1-phosphate

Relatively little is known about the regulation of ceramide kinase (cloned in 2002, Sugiura *et al.*, 2002) and, therefore, it is difficult to assess whether some effects originally ascribed to ceramide may be due to activation of ceramide kinase and conversion of ceramide to C1P. The role of C1P in inflammation is consequently less explored compared with other sphingolipids but, based on recent studies, is probably predominantly via activation of $cPLA_2$ (Pettus *et al.*, 2004; Lamour and Chalfont, 2005). This is likely via a direct interaction with a Ca^{2+}-dependent phospholipid binding domain (Subramanian *et al.*, 2005), although has also been reported to occur via interaction with protein kinase C isoforms (Nakamura *et al.*, 2006). Studies indicating the importance of C1P in inflammatory processes have demonstrated that exogenously added C1P can lead to PGE_2 production (Pettus *et al.*, 2003a). In addition, IL-1β stimulation in A549 cells leads to C1P production and subsequent PGE_2 up-regulation. This up-regulation is inhibited by RNAi knock-down of ceramide kinase (Pettus *et al.*, 2003a). Interestingly, with regard to the PGE_2 up-regulation, there appears to be some synergy with S1P. This coordinated response is demonstrated by C1P activation of PLA_2 occurring simultaneously with S1P-induced COX-2 up-regulation (Pettus *et al.*, 2005). The increase in arachidonic acid is therefore more effectively utilized for eicosanoid synthesis.

Mast cell degranulation mediates inflammation through releasing bioactive mediators from vesicles leading to the recruitment of inflammatory cells and subsequent release of further cytokines and chemokines. In a mast cell model (RBL-2H3 cells), C1P was observed to stimulate degranulation in a Ca^{2+}-dependent manner (Mitsutake *et al.*, 2004). This Ca^{2+} dependence may be required by ceramide kinase for activation, with calmodulin as the likely Ca^{2+} sensor (Mitsutake and Igarashi, 2005). Overexpression of ceramide kinase enhances degranulation and a novel inhibitor of ceramide kinase, K1 (Kim *et al.*, 2005b) results in an inhibition of degranulation. As degranulation involves the fusion of vesicles with the plasma membrane, it is suggested that the conversion of ceramide to C1P may alter the sphingolipid balance in the membrane and result in enhanced vesicle fusion (Mitsutake *et al.*, 2004). Recent evidence using a ceramide kinase –/– mouse line has, however, suggested that C1P may not be a major pathway of mast cell degranulation. In these animals various indicators of mast cell function were unchanged (Graf *et al.*, 2008). The relative role of C1P in mast cell functions therefore remains controversial.

Sphingosine 1-phosphate

With a better characterized mode of action compared with ceramide and C1P, investigation of the roles of S1P has provided more direct evidence for its relative importance in inflammation. However, the effects of S1P may differ dependent on the environment. Intracellular SK activation can produce S1P in response to specific stimuli or, in the case of cells circulating in the vascular system, the occurrence of S1P in plasma means that synthesis of S1P (and therefore activation of SK) is not necessarily required to produce effects. It is possible that regulation of the presentation of S1P receptors in the membrane, rather than S1P availability, may be involved. In relation to the role of S1P in inflammation, there is also a degree of cell type specificity.

In mast cells, S1P is now known to have an important role during activation and subsequent development of the inflammatory response (Olivera, 2008). Antigen engagement of the high-affinity receptor for IgE on mast cells results in the activation of SK and production of S1P (Choi *et al*, 1996; Prieschl *et al.*, 1999). Studies in knockout mouse lines suggest that the SK2 isoenzyme is predominantly responsible for S1P production in this case (Olivera *et al.*, 2007), probably via a signalling cascade involving activation of the non-receptor tyrosine kinase, Fyn (Olivera *et al.*, 2006). A deficiency in SK2 results in a decreased degranulation and decreased production of eicosanoids and cytokines highlighting the importance of S1P production. Following activation of SK2, the resultant S1P produced is exported from the mast cells via an ATP-binding cassette family of transport proteins (Mitra *et al.*, 2006). S1P is likely to have both autocrine effects on the mast cells in addition to paracrine effects on other cells recruited to the site of mast cell acti-

vation. Mast cells express $S1P_1$ and $S1P_2$ receptors and these mediate different but important effects of mast cell activation (Jolly *et al.*, 2004). $S1P_1$ is involved in mast cell migration while $S1P_2$ is important in degranulation. Given the critical role of mast cells in pathophysiology, it is not unexpected that the role of S1P in mast cell function translates into several different areas of clinical relevance. These are discussed later in the context of therapeutic intervention.

In other cell types, S1P is also involved as part of the inflammatory process, particularly in response to the cytokine TNF. In L929 fibroblasts and A549 lung epithelial cells, the TNF-induced up-regulation of COX-2 expression and subsequent PGE_2 production was dependent on activation of SK (Pettus *et al.*, 2003b). SK1, but not SK2, was required for COX-2 induction. Involvement of S1P receptors was not assessed. In macrophages, COX-2 can be induced by stimulation with LPS, which activates a well-characterized antibacterial pathway leading to activation of NF-κB. In the RAW macrophage cell line this pathway was also found to be dependent on SK1 through up-regulation of SK1 transcription (Hammad *et al.*, 2008), suggesting that the role of S1P in COX-2 induction is not restricted to TNF. S1P can also regulate upstream of COX-2 in an inflammatory response. In A549 lung epithelial cells, $cPLA_2$ is activated by S1P to produce arachidonic acid (Chen *et al.*, 2008) via an $S1P_3$-mediated increase in $[Ca^{2+}]_i$ and RhoA activation.

Some recent *in vivo* studies are beginning to highlight the importance of S1P and S1P receptor signalling in pathological inflammation. For example, pathological angiogenesis in the retina gives rise to abnormal and dysfunctional blood vessels that grow into the vitreous, which under normal conditions is avascular (Saint-Geniez and D'Amore, 2004). This is common in diabetic retinopathy. In pathological angiogenesis induced by hypoxia in mouse retina, activation of the $S1P_2$ receptor on corneal endothelial cells is essential for up-regulation of COX-2-induced inflammation and drives the neovascularisation of avascular areas (Skoura *et al.*, 2007). In $S1P_2$–/– mice this inappropriate angiogenesis was significantly inhibited and correlated directly with decreased COX-2 expression. The inflammation induced by S1P relates directly to retinopathy in pathological conditions and suggests $S1P_2$ receptor as a novel therapeutic target. Another recent study has examined the possible role of S1P in pathological disruption of the lung epithelial barrier integrity (Gon *et al.*, 2005). Acute lung injury is characterized by an inflammation resulting in damage to the endothelial and epithelial cell barriers. This leads to the filling of alveolar spaces with fluid and inflammatory cells and ultimately results in respiratory failure. The epithelial tight junctions are therefore essential to the permeability barrier in the lung. Exposure of mouse airways to S1P *in vivo* leads to a disruption of the epithelial barrier and subsequently gives rise to lung oedema, an effect that is synergistic with TNF. In $S1P_3$–/– mice, S1P did not disrupt the tight junctions of the epithelial barrier suggesting an important role for $S1P_3$ in this pathological effect (Gon *et al.*, 2005). Whether these S1P effects in whole lung are via up-regulation of COX-2 or $cPLA_2$ (as previously observed in the A549 lung epithelial cell line; Pettus *et al.*, 2003b; Chen *et al.*, 2008) is not yet clear. The $S1P_3$ receptor may be a target with therapeutic benefit to prevent inflammation following lung injury.

SPC, structurally related to S1P, has affinity for S1P receptors (although lower than S1P) and initial studies have suggested that it may also be pro-inflammatory. In vascular smooth muscle cells, SPC activates p38MAPK (Mathieson and Nixon, 2006), associated with inflammation, and results in the release of TNF (Nixon *et al.*, 2008). Also in keratinocytes, SPC induces release of TNF (Imokawa *et al.*, 1999). Whether these effects are via S1P receptor activation is unknown.

Potential therapeutic intervention in sphingolipid-induced inflammation

It is clear from many recent studies that sphingolipids can be intimately involved in the onset and maintenance of inflammation. This indicates that the targeting of sphingolipid actions as part of an anti-inflammatory therapeutic strategy would be beneficial in a number of different clinical conditions. While this strategy is at an early stage of development, studies have begun to demonstrate the potential importance of these lipids as an effective area for pharmacological intervention. There are now several compounds that have been developed, which can pharmacologically manipulate different components of the sphingomyelin cycle, and in particular S1P synthesis and S1P receptors (recently reviewed in Huwiler and Pfeilschifter, 2008). Only a few of these compounds have been examined in the context of a therapeutic benefit in inflammation. The remainder of this review will concentrate on those areas where studies in animal models of specific disease states have used existing drugs or novel therapeutic agents that mediate an anti-inflammatory action via regulation of sphingolipids (summarized in Table 1).

FTY720

The most high-profile drug that regulates sphingolipid effects on inflammation and has been assessed *in vivo* is the immunosuppressant FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl] propane-1,3-diol), a fungal metabolite. FTY720 is phosphorylated *in vivo* (Billich *et al.*, 2003) and FTY720-P (structure shown in Figure 1) is an effective modulator of the immune system in transplant models and in renal transplant in humans (Mansoor & Melendez, 2008). Its mode of action is not via the typical immunosuppressant actions of existing drugs (inhibition of T-cell function), but from a sequestration of lymphocytes to the lymph nodes. This reduces the number of T-cells circulating between lymph nodes and the peripheral site of tissue inflammation (Pinschewer *et al.*, 2000). Subsequent studies have now demonstrated that FTY720-P has high affinity for four S1P receptor subtypes: $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$. Moreover, FTY720 is phosphorylated by SK *in vivo* (Billich *et al.*, 2003). Using conditional knockout mice, it has been demonstrated that SK2 is the isoenzyme involved in this phosphorylation (Zemann *et al.*, 2006). It is now clear that the mechanism of action of FTY720-P is via binding to $S1P_1$ receptors on lymphocytes (Brinkmann *et al.*, 2002; Mandala *et al.*,

Table 1 The potential role of sphingolipids and drug targets in inflammatory diseases

Tissue	*Bronchioles*			*Intestine*			*Retina*	*Lung*
Disease Pathology	Asthma Cytokine-induced hyperresponsiveness			Colitis Inflammation of mucosa			Diabetic retinopathy COX-2-induced vascularization	Acutelung injury Cytokine-induced oedema
Cell type	T-cells (Th2)	mast cells	dendritic cells	T-cells/macrophages			Corneal endothelial cells	Lung epithelial cells
Sphingolipid target in inflammation	S1PR	SK1	S1PR	$S1P_1R$	acid SMase	SK1	$S1P_2R$	$S1P_3R$
Drugs assessed in *in vivo* models	FTY720	SK-I DMS	FTY720	KRP-203	SMA-7	ABC294640 ABC747080	ND	ND

Summary of studies examining the *in vivo* involvement of sphingolipids in inflammation associated with different disease models. Drugs targeting sphingolipids, where determined, are also shown. COX-2, cyclooxygenase-2; DMS, N,N-dimethylsphingosine; ND, not determined; S1PR, sphingosine 1-phosphate receptor; SK, sphingosine kinase.

2002). Activation of this S1P receptor subtype is essential to allow egress of lymphocytes from the lymph nodes. Although FTY720-P is an agonist with nanomolar affinity for the $S1P_1$ receptor, the mechanism of action is possibly not via its agonist properties. The important mode of action in this case may be via a down-regulation of the $S1P_1$ receptor on lymphocytes (Matloubian *et al.*, 2004). Therefore, by preventing recycling of the receptor to the plasma membrane, FTY720 effectively prevents activation of the $S1P_1$ receptor and inhibits lymphocyte egress from lymph nodes. This subsequently decreases T-cell-induced activation of the inflammatory response. Although this drug is effective as an immunosuppressant in transplantation as well as having therapeutic potential in other areas, such as multiple sclerosis and cancer (Hiestand *et al.*, 2008), its use as an anti-inflammatory drug is still at a preliminary stage of investigation. The possible roles of FTY720 as a drug in certain inflammatory diseases, together with other potential agents that modulate sphingolipids, are discussed below with reference to asthma and inflammatory bowel disease (IBD). Both these disease states have important inflammatory components. The remainder of this review will concentrate on drugs that directly block the anti-inflammatory actions of sphingolipid effects. Other potential therapeutic treatments for these diseases will not be covered and readers are referred to specialist reviews on asthma and IBD.

Sphingolipid therapeutic targets in asthma

Asthma is characterized by a bronchial hyperresponsiveness of airway smooth muscle cells and a proliferative remodelling of the airways (Halayko and Amrani, 2003). These effects are ultimately the result of an initial inflammatory cell infiltration of the airways following antigen challenge. This results in an increase in release of cytokines and chemokines from various cell types, such as macrophages and mast cells. Asthma is associated with increased TNF levels in bronchioalveolar lavage (Hallsworth *et al.*, 1994). In addition, TNF has a prominent role in airway hyperresponsiveness (Hunter *et al.*, 2003) and, within a pathophysiological concentration range found in asthmatic bronchioalveolar lavage, can induce DNA synthesis in ASM cells (Stewart *et al.*, 1995). It should be noted that while the role of inflammation in asthmatic airway remodelling has been indicated in animal models, this remains to be established in humans (Tang *et al.*, 2006). Both the hyperrresponsiveness (Roviezzo *et al.*, 2007) and proliferation of airway smooth muscle (Waters *et al.*, 2003) have been shown in some model systems to involve sphingolipids and in particular S1P (Ammit *et al.*, 2001; Jolly *et al.*, 2002). The individual mechanisms involved, however, can be secondary to the initial inflammation. For example, the proliferative effect may be via a transactivation of the PDGF receptor by the $S1P_1$ receptor and subsequent MAPK activation (Waters *et al.*, 2003). The hyperresponsiveness may involve S1P-induced activation of the RhoA/Rho-kinase pathway leading to an increased Ca^{2+} sensitivity of the contractile myofilaments (Kume *et al.*, 2007). The source of sphingolipid, and more specifically in this case S1P, is likely to be either directly released from activated mast cells (Mitra *et al.*, 2006), or the result of activation of the SMase pathway by TNF leading to *de novo* production. Indeed, S1P levels are increased in bronchioalveolar lavage from asthmatics following antigen challenge (Ammit *et al.*, 2001), providing evidence that increased S1P release/production in asthma is directly linked to the inflammation. It is therefore not surprising that sphingolipids are potential anti-inflammatory targets in asthma.

A few studies have now examined the effectiveness of FTY720 in asthmatic models. Using an *in vivo* Th2 cell transfer mouse model, oral FTY720 treatment decreased infiltration of pro-inflammatory eosinophils and T-cells into the airway mucosa (Sawicka *et al.*, 2003). FTY720 also had similar effects on a Th1 cell transfer mouse model. Both Th1 and Th2 cells express $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ receptor isoforms. Th2 cells are the T-cell subset that are considered to be predominantly responsible for initiating the inflammatory response in asthma via activation of oesinophils (Anderson and Coyle, 1994). Th2-mediated inflammation is causally related to several characteristics of asthma, such as airway hyperrespon-

siveness. Importantly, when the Th2 transfer mice were challenged with ovalbumin, FTY720 also decreased the airway hyperresponsiveness to agonist challenge. In this model, the mode of action of FTY720 is not clear and whether this occurs via down-regulation of the $S1P_1$ receptor is not yet established. It appears unlikely to be an effect on mast cells as mast cell degranulation is via $S1P_2$ receptor (Jolly *et al.*, 2004), the S1P receptor isoform that does not bind FTY720P. Interestingly, *in vitro* FTY720 (unphosphorylated) inhibited $cPLA_2$ activation independently of S1P receptor in RBL-2H3 mast cells (Payne *et al.*, 2007). This effect was not observed with phosphorylated FTY720. Although it is not clear whether this occurs *in vivo*, it raises another possible mechanism for the FTY720-mediated anti-inflammatory effect in asthma.

Another study also examining the role of sphingolipids in mouse models of asthma has demonstrated that, while aerosol administration of FTY720 decreases Th2 cell-mediated inflammation and bronchial hyperresponsiveness, T-cell retention in the lymph nodes was not observed (Idzko *et al.*, 2006). In this case the mode of action was an inhibition of dendritic cell migration. Dendritic cells, which express all five isoforms of the S1P receptor, present antigen to T-cells and initiate an immune response. Inhibition of dendritic cell migration subsequently decreased formation of Th2 cells in lymph nodes and thereby prevents airway inflammation. Therefore, in at least some asthmatic models, the mechanism of action of FTY720 may occur via mechanisms other than preventing T-cell egress from the lymph nodes. Whether this action is via direct agonist properties of FTY720 or is via down-regulation of S1P receptors remains to be determined.

Other potential therapeutic targets for asthma within the sphingomyelin cycle have also been examined. Results obtained using *in vivo* mouse models have indicated the possible benefits of SK inhibitors. In ovalbumin-challenged mice intraperitoneal administration of the selective SK inhibitor, N,N-dimethylsphingosine, decreased the infiltration of pro-inflammatory cells, such as eosinophils and macrophages and also decreased Th2 cell-mediated cytokine release (Lai *et al.*, 2008). Importantly, the methacholine-induced airway hyperresponsiveness was also reduced by SK inhibition. To ensure that these effects were via SK inhibition, siRNA knockdown of SK1 in the asthmatic mice has similar effects. IgE levels were also lowered suggesting an inhibition of mast cell degranulation. A similar study has also shown that N, N-dimethylsphingosine and SK-I (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole) administered via inhalation also decreased airway inflammation and hyperresponsiveness in ovalbumin-sensitized mice (Nishiuma *et al.*, 2008). The increased S1P concentration in the bronchioalveolar lavage of these animals was decreased to basal levels by inhibition of SK, suggesting that the effects are directly linked to decreased S1P concentrations. These studies further indicate the potential of the SK/S1P pathway in providing a promising therapeutic target in asthma. Clarification on the potentially multiple mechanisms with different models will lead new directions for asthma therapy.

Sphingolipid therapeutic targets in IBD

IBD is a collective definition for several different diseases, principally ulcerative colitis and Crohn's disease, which are the result of damage to the intestinal mucosa. This damage is caused by cellular inflammation due to an imbalanced cytokine production from dysfunctional T-cells (Braegger and MacDonald, 1994). Specifically, regulatory T-cells do not respond to normal (effector) T-cell stimulation, but they prevent inflammation by the production of suppressor cytokines, including IL-10 and transforming growth factor-β (Maloy and Powrie, 2001). In a recent study the therapeutic potential of FTY720 in a chemically induced mouse model of colitis was examined (Daniel *et al.*, 2007). Treatment with FTY720 significantly reduced all clinical and pathological indications of intestinal inflammation in this model. This was correlated with a decrease in inflammatory cytokines, for example, TNF, released from effector T-cells with a concomitant increase in the release of IL-10 and transforming growth factor-β from regulatory T-cells. The exact signalling mechanisms of these effects are not clear, and the S1P receptor subtypes involved on T-cells are not yet defined. However, it does indicate further the therapeutic potential for FTY720.

Another study has also examined regulation of S1P receptors in models of colitis. The effects of a novel S1P agonist KRP-203, which has structural homology to FTY-720 and is similarly phosphorylated *in vivo*, was examined in an IL-10–/– mouse model of colitis (Song *et al.*, 2008). KRP-203 has similar agonist efficacy to FTY720 at $S1P_1$ and $S1P_4$ receptors but has much lower efficacy at $S1P_3$. Oral administration of KRP-203 resulted in a decrease in the pathological symptoms of colitis, including decreased weight loss and normal intestinal wall thickness. This was attributed to a sequestration of lymphocytes to lymph nodes. KRP-203 therefore produces its action by preventing infiltration of lymphocytes into the intestinal mucosa and results in a decreased release of cytokines, including TNF. Although it is not known if KRP-203 acts to down-regulate $S1P_1$ receptor, similar to FTY720, the results indicate that this may be the case. Certainly, its low efficacy at $S1P_3$ receptors suggests that $S1P_1$ and/or possibly $S1P_4$ are involved.

In addition to S1P receptors, the *de novo* production of S1P via SK activation has also been examined as a potential therapeutic target in IBD. This has been verified by a recent study using SK1–/– mice (Snider *et al.*, 2009). SK–/– mice treated with dextran sulphate sodium (DSS) to induce colitis had significantly less intestinal damage compared with controls. Also, unlike DSS-treated controls, SK1–/– mice did not display a systemic inflammatory response and did not have any colonic COX-2 induction. Pharmacological evidence has further validated SK1 inhibition as a potential therapeutic target in IBD. In the DSS-induced colitis mouse model, two orally active novel SK inhibitors (ABC294640 and ABC747080), which were effective *in vitro*, were examined (Maines *et al.*, 2008). Both inhibitors decreased the development and progression of colitis, including less colon shortening and colonic inflammation. Inflammatory cytokines, such as TNF IL-1β and IL-6, were reduced. SK inhibitors may therefore represent another potential target in IBD.

Evidence that other points on the sphingomyelin cycle may also be therapeutic targets in colitis has been demonstrated by a study using a novel acid SMase inhibitor. SMA-7 inhibited LPS-induced activation of NF-κB and release of the pro-inflammatory cytokines TNF, Il-1β and IL-6 in macrophages (Sakata *et al.*, 2007). This was correlated with decreased cera-

mide production. In an *in vivo* chemically induced mouse model of colitis, oral administration of SMA-7 resulted in decreased cytokine levels in the colon and lower severity of colonic injury. Whether the inhibition of ceramide is the key mechanism in this effect, or a decreased ceramide production prevents further downstream conversion to sphingosine and S1P, is not known.

Conclusion

In recent years, it has been established that sphingolipids are important mediators of cell function and are fundamentally involved in many cellular processes. *In vitro* evidence that sphingolipids can modulate inflammation and may be integral in the up-regulation of inflammatory pathways, such as the induction of COX-2, has been growing rapidly. Due to the interdependent nature of the sphingomyelin cycle, effects attributed to one lipid may be due to conversion or breakdown to another lipid mediator. For example, effects attributed to ceramide could be due to conversion to C1P, or generation of sphingosine and ultimately S1P. Therefore, while there seems little doubt that sphingolipids are important in inflammation, the exact modes of action in some cases are not entirely clear. This is perhaps less so with studies examining the S1P receptor agonist, FTY720. This drug has validated a new target with a better defined mode of action, which has not yet been fully assessed in clinically relevant situations using *in vivo* models of inflammatory diseases. Recent studies suggest that S1P receptors are a promising therapeutic target, particularly in asthma and IBD. Further research is required to identify exactly which S1P receptors are important in different cell types to regulate inflammation in disease conditions. Experiments can also better define the pharmacokinetics and pharmacodynamics of such S1P receptor agonists *in vivo*. It is not completely clear if all the observed effects occur predominantly through receptor down-regulation, thereby preventing receptor recycling or whether some are via S1P receptor activation and resultant signalling cascades. However, there is little doubt that further development of similar drugs directed towards the modulation of sphingolipids, and in particular S1P receptors, will provide a new generation of drugs to counteract pathological inflammation.

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