CMLS, Cell. Mol. Life Sci. 58 (2001) 2053–2068
1420-682X/01/142053-16 $ 1.50 + 0.20/0
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CMLS Cellular and Molecular Life Sciences

Review

Sphingolipids in mammalian cell signalling

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Received 16 May 2001; received after revision 29 June 2001; accepted 3 July 2001

Abstract. Sphingolipids and their metabolites, ceramide, sphingosine and sphingosine-1-phosphate, are involved in a variety of cellular processes including differentiation, cellular senescence, apoptosis and proliferation. Ceramide is the main second messenger, and is produced by sphingomyelinase-induced hydrolysis of sphingomyelin and by de novo synthesis. Many stimuli, e.g. growth factors, cytokines, G protein-coupled receptor agonists and stress (UV irradiation) increase cellular ceramide levels. Sphingomyelin in the plasma membrane is located primarily in the outer (extracellular) leaflet of the bilayer, whilst sphingomyelinases are found at the inner (cytosolic) face and within lysosomes/endosomes. Such cellular compartmentalisation restricts the site of ceramide production and subsequent interaction with target proteins. Glycosphingolipids and sphingomyelin together with cholesterol are major components of specialised membrane microdomains known as lipid rafts, which are involved in receptor aggregation and immune responses. Many signalling molecules, for example Src family tyrosine kinases and glycosylinositolphosphate-anchored proteins, are associated with rafts, and disruption of these domains affects cellular responses such as apoptosis. Sphingosine and sphingosine-1-phosphate derived from ceramide are also signalling molecules. In particular, sphingosine-1-phosphate is involved in proliferation, differentiation and apoptosis. Sphingosine-1-phosphate can act both extracellularly through endothelial-differentiating gene (EDG) family G protein-coupled receptors and intracellularly through direct interactions with target proteins. The importance of sphingolipid signalling in cardiovascular development has been reinforced by recent reports implicating EDG receptors in the regulation of embryonic cardiac and vascular morphogenesis.

Key words. Sphingomyelin; ceramide; sphingosine-1-phosphate; signal transduction; lipid raft; caveolae.

Introduction

Lipids are integral structural components of cell membranes, which through their ability to form a bilayer produce a permeability barrier between extracellular and intracellular compartments, a function essential for cell survival. In addition, lipids are essential for signal transduction in response to agonist stimulation as their hydrolysis produces bioactive molecules known to trigger many downstream signalling cascades. The first evidence for such a signalling role came in the 1970s with the discovery of the phosphoinositide (PI) cycle. Subsequently, many studies have shown that a primary event following receptor activation is hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by PI-phospholipase C (PI-PLC), releasing the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 modulates intracellular calcium levels by controlling calcium channels at both the plasma membrane and endoplasmic reticulum [1, 2] and DG binds to and activates protein kinase C (PKC) [3] so initiating a distinct and separate signalling cascade. Further studies have shown the production of many bioactive lipids generated by receptor-mediated hydrolysis of glycerophospholipids such as

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phosphatidic acid (PA) produced by PLD acting on phosphatidylcholine (PC) [4] or by DG kinases phosphorylating DG [5]. Arachidonic acid produced by the action of PLA_2s is also recognised as an important signalling molecule as well as being the precursor of a diverse group of bioactive compounds, the eicosanoids [6]. More recently, 3-phosphoinositides generated following growth factor and G protein-coupled receptor activation by the action of PI 3-kinases (PI3Ks) on inositol phospholipids have been recognised as important signalling lipids, [7]. One target of these lipid messengers is PKB/Akt an important cell survival pathway [8]. In addition to glycerolipids, a second class of lipids – sphingolipids – are now known to act as a reservoir of signalling molecules [9–11]. Sphingolipids, of which there are over 300, are found in all eukaryotic cells and are enriched in plasma membranes, Golgi membranes and lysosomes [11, 12]. In 1986, the sphingolipid derivative sphingosine was shown to inhibit PKC [13], demonstrating that this class of lipid was involved in cell signalling. Another sphingolipid-derived second messenger is ceramide, a product of sphingomyelin. We now know that many stress stimuli (e.g. cytokines, cytotoxic agents, environmental stress and injury or infection) increase cellular ceramide levels through hydrolysis of sphingomyelin [14] and there is substantial evidence that ceramide is involved in apoptosis [15, 16].

Although the major role of glycerolipids and sphingolipids in signal transduction appears to be as a reservoir of lipid-derived second messengers, a growing body of evidence points to a signalling role of sphingolipids as structural components of cell membranes. Recently, specialised membrane microdomains enriched in cholesterol and sphingomyelin have been recognised as centres for the organisation of signalling molecules. These domains, known as lipid rafts [17], are important for immune cell responses [18], and may be involved in G protein-coupled receptor [19] and growth factor receptor [20] signalling. Disruption of lipid rafts by depletion of cholesterol inhibits T cell receptor signalling, demonstrating the importance of these structures [21]. Additionally, the majority of lipid second messengers are extremely hydrophobic and will remain compartmentalised within the membrane; consequently, the site of phospholipid hydrolysis would be expected to influence the cellular response. Within the plasma membrane, phospholipids are asymmetrically arranged such that cholinephospholipids, PC and sphingomyelin (up to 90% dependent on cell type) are predominantly found in the outer (extracellular) leaflet and aminophospholipids, phosphatidylserine, phosphatidylethanolamine and probably PI are located mainly in the inner (cytoplasmic) leaflet [22]. Membrane asymmetry is actively maintained by at least three distinct activities: aminophospholipid translocase, ATP-dependent floppase and lipid scramblase [reviewed in ref. 23] and loss of asymmetry, particularly the appearance of phosphatidylserine on the outer surface is associated with physiologic and pathologic processes, such as thrombosis [23] and apoptosis [24, 25]. The asymmetric distribution of phospholipids within the plasma membrane could influence signal transduction by limiting the access of phospholipases to their substrate. Asymmetry may also introduce specificity to cellular responses by requiring a mechanism to present phospholipids to their respective phospholipases. Evidence for such a mechanism has recently been reported, with redistribution of sphingomyelin to the inner leaflet in response to CD95 being necessary for ceramide generation [26].

Sphingolipids are now widely accepted to play an important role in cell signalling. Clearly, ceramide is involved in stress responses leading to apoptosis in many cell types. However, there is also evidence that changes in sphingomyelin levels resulting in membrane perturbation may regulate signalling pathways. In this review, we shall discuss the evidence for the role of sphingolipids in mammalian cell signalling and attempt to describe the mechanisms by which this diverse class of lipids elicit a cellular response.

Sphingolipids

Sphingolipids are characterised by their sphingoid backbone. In mammalian cells, sphingosine is the most common sphingoid base, while in yeast and plant cells, phytosphingosine is more common. Sphingolipid biosynthesis (fig. 1) begins with the condensation of serine and palmitoylCoA forming 3-ketosphingosine which in turn undergoes reduction to dihydrosphingosine. A fatty acyl group is added by an amide linkage to form dihydroceramide, which is converted directly to ceramide, the precursor of all sphingolipids, by the introduction of a trans double bond between carbons 4 and 5 of the sphingoid base [27]. Different headgroups may then be added to ceramide to form more complex sphingolipids, the simplest of which is ceramide-1-phosphate formed by ceramide kinase. More complex headgroups include β-glycosidically linked glucose- or galactose-cerebrosides, the addition of a sulphate group to galactosylceramide yields sulphatides and di-, tri- and tetra-glycosylceramides are known as glycosphingolipids. Gangliosides are a subclass of glycosphingolipids identified by the presence of sialic acid in the carbohydrate headgroup [11]. The addition of phosphorylcholine to ceramide, transferred from PC by sphingomyelin synthase, forms sphingomyelin [27]. Lyso-sphingolipids, *N*-deacylated derivatives such as 1-galactosylsphingosine, glucosylsphingosine, sphingosine-1-phosphate and lysosphingomyelin are also found. These sphingolipids are present at very low concentrations but may have important signalling effects either as second messengers, e.g. sphingosine-1-phos-

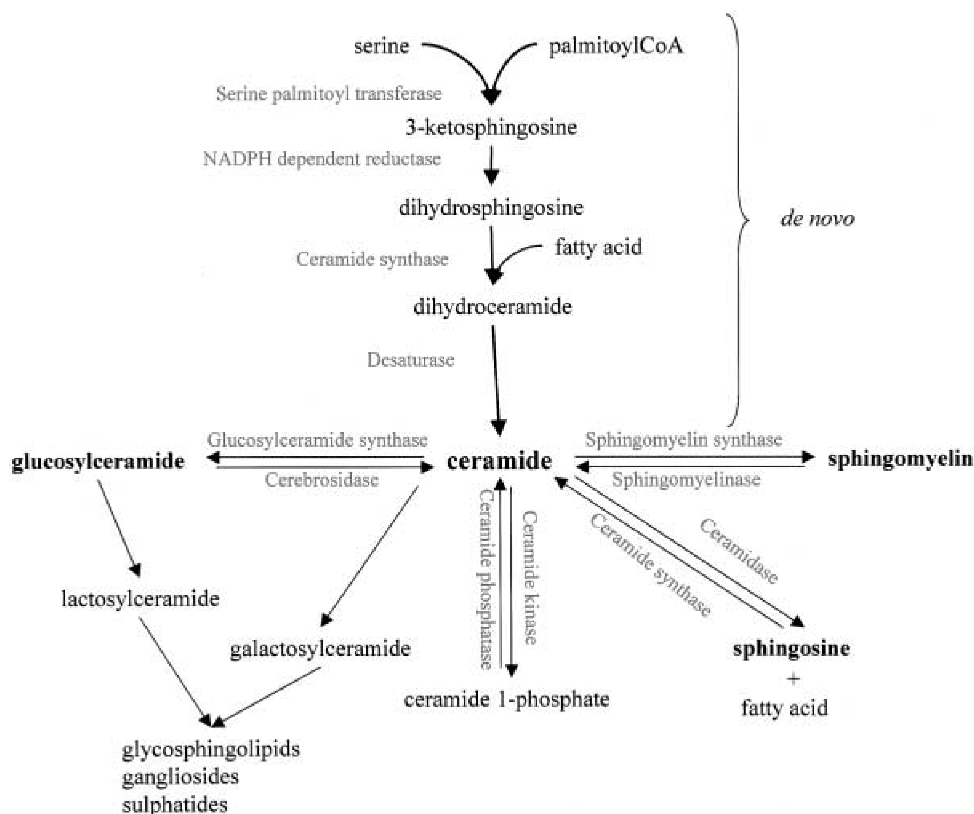

Figure 1. Sphingolipid biosynthesis. De novo synthesis of ceramide takes place in the endoplasmic reticulum. The synthesis of sphingomyelin, glucosylceramide and glycosphingolipids from ceramide occurs in the Golgi apparatus, whilst degradation of glycosphingolipids and sphingomyelin to ceramide and sphingosine occurs in lysosomes.

phate, or through their lytic and membrane-destabilising effects [28]. For a recent comprehensive review of the structure, function and biosynthesis of complex sphingolipids see Huwiler et al. [11].

Sphingomyelin

Sphingomyelin is made up of a long-chain sphingoid base, predominantly D-*erythro*-sphingosine in mammalian cells, an amide-linked acyl chain which may be of long (22:0, 24:0 and $24:1^{\Delta 15(c)}$) or intermediate (16:0 and 18:0) length [29] and a phosphorylcholine headgroup (fig. 2). The acyl chain composition varies between tissues; for example in the brain, white matter sphingomyelin contains predominantly nervonic acid ($24:1^{\Delta 15(c)}$) whilst in grey matter, oleic acid (18:0) predominates [30]. In contrast, a mixed population of sphingomyelins appears to be present in non-neuronal cells [29]. Differences in the fatty acid composition of the amide-linked acyl side chain in sphingomyelins may have effects on membrane properties. For example, a predominance of saturated acyl chains contributes to the high phase transition temperatures characteristic of sphingolipids [31]. In addition, the fatty acid composition may affect the interaction of sphingomyelin with cholesterol [32], which in turn could affect membrane composition.

Subcellular localisation

Sphingomyelin is present in most eukaryotic cell membranes. Subcellular fractionation studies indicate that greater than 50% of cellular sphingomyelin is located in the plasma membrane [reviewed in ref. 33]. Treatment of cells with bacterial sphingomyelinases suggests that the majority of plasma membrane sphingomyelin is in the outer membrane leaflet. For example, approximately 90% of cellular sphingomyelin was hydrolysed within 25 min by bacterial sphingomyelinase in human lung fibroblasts [34]. However, there also appears to be a pool of sphingomyelin located at the cytosolic face of the plasma membrane that is available for receptor-stimulated hydrolysis by neutral sphingomyelinases [35]. There is also evidence that in response to apoptotic stimuli or elevated intracellular calcium, phospholipid scrambling occurs such that sphingomyelin moves from the outer to the inner plasma membrane leaflet [26]. Such a reorientation renders sphingomyelin susceptible to degradation by cy-

tosolic neutral sphingomyelinase and is essential for the generation of ceramide and the morphological changes, e.g. membrane blebbing, associated with apoptosis [26]. In contrast, another study has shown that the cytokine CD95 induces translocation of an acid sphingomyelinase to the outer plasma membrane leaflet and that ceramide is formed at the extracellular surface [36]. The fact that cells expend energy to maintain plasma membrane phospholipid asymmetry [23] and have developed multiple and complex mechanisms to regulate sphingomyelin degradation further emphasises the importance of this sphingolipid in normal cell function.

Biosynthesis

Ceramide forms the basic structural backbone of sphingolipids analogous to DG in glycerolipids (fig. 2). The de novo synthesis of ceramide takes place in the endoplasmic reticulum and conversion to sphingomyelin by sphingomyelin synthase (SMS) takes place in the Golgi apparatus [11]. Sphingomyelin is then transported through an exocytic pathway to the plasma membrane. Localisation of sphingomyelin at the outer leaflet of the plasma membrane appears to be due to the topography of SMS, found at the luminal as opposed to the cytosolic side of the Golgi membrane, as there is no evidence for a sphingomyelin-selective flippase at the plasma membrane. There is evidence that sphingomyelin synthesis can occur at other sites within the cell, because SMS activity has been found at the plasma membrane [37–39], in the trans-Golgi network [40] and in rat liver nuclear membranes [41].

Sphingomyelin synthase

Phosphatidylcholine: ceramide phosphocholine transferase (SMS) transfers the phosphorylcholine headgroup from PC to ceramide, forming sphingomyelin and releasing DG (fig. 3). This activity places SMS at a potentially important regulatory position in signalling pathways that utilise ceramide and DG as second messengers because it could act to switch an anti-mitogenic signal (ceramide) to a mitogenic signal (DG). Indeed, in a recent report with a human T cell line, the ratio of DG/ceramide and PC/sphingomyelin was high in proliferating cells and decreased in growth-arrested cells [42]. However, this was due to increased de novo synthesis of DG and PC and SMS activity was found only to increase in cells under-

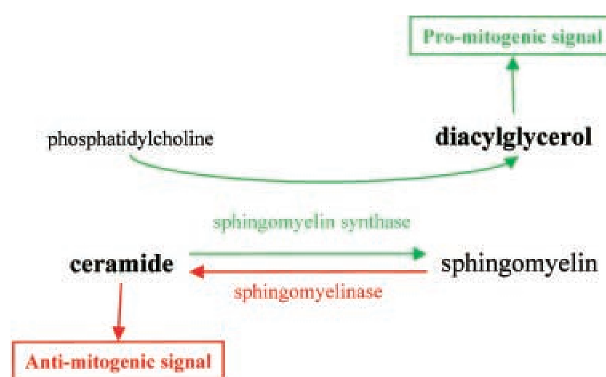

Figure 3. Regulation of mitogenesis by ceramide and diacylglycerol (DG). Stimuli that increase sphingomyelin synthase activity will increase the levels of DG at the expense of ceramide, so favouring the mitogenic signalling pathways in which DG is a second messenger. Activation of sphingomyelinase will increase ceramide levels and initiate an anti-mitogenic signal.

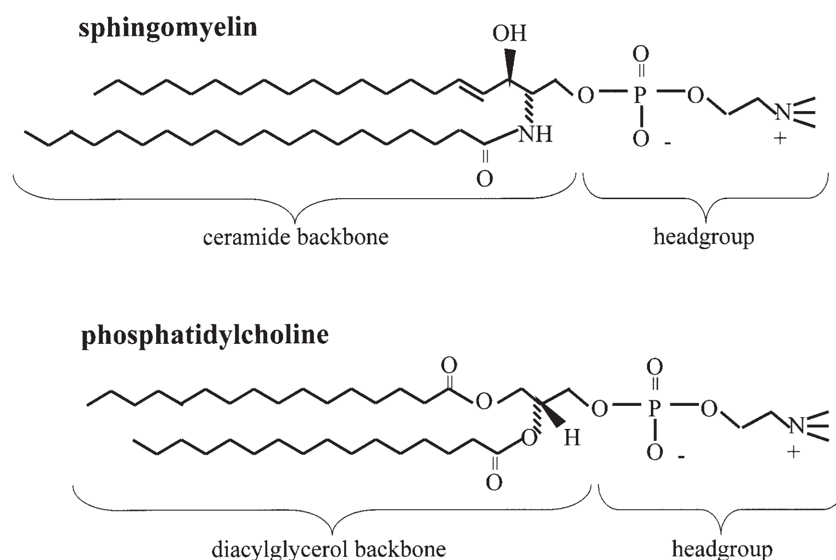

Figure 2. Molecular structure of sphingomyelin and phosphatidylcholine. Ceramide forms the backbone of sphingolipids analogous to diacylglycerol in phospholipids. The headgroup, phosphocholine in this representation, determines the type of sphingolipid or phospholipid.

going apoptosis [42]. Although many studies have investigated the subcellular localisation of SMS [37–41], few have investigated the role of this enzyme in signal transduction. In primary astrocytes, basic fibroblast growth factor (bFGF) induces a decrease in ceramide levels and an increase in sphingomyelin synthesis through activation of SMS in a cellular compartment remote from the Golgi apparatus [43]. D609, an inhibitor of PC-PLC [44] that also inhibits SMS [34], prevented bFGF-induced astrocyte proliferation, suggesting that a non-Golgi SMS may play a role in regulation of cell growth. This study raises the intriguing possibility that the reported PC-PLC activity involved in growth factor regulation of cell proliferation [45–47] is at least in part a SMS, as proposed by Luberto and Hannun [34]. There are many similarities between PC-PLC and SMS. Both enzymes consume PC and produce DG, both operate at neutral pH and appear to be plasma membrane associated [34]. Furthermore, elevated PC-PLC activity has been reported in transformed cells [48] and SMS activity is enhanced in SV40-transformed human lung fibroblasts [34] and hepatomas [34, 49]. The resolution of whether these are the same or different enzymes awaits their cloning and characterisation. The role of enhanced SMS activity in transformed cells is not clear. In SV40-transformed human lung fibroblasts, activation of NFκB correlated with the conversion of ceramide to sphingomyelin, suggesting a role for SMS in this pathway [50] and, as described above, increased SMS activity may be important for bFGF-induced astrocyte proliferation [43]. However, whether SMS affects cellular responses by acting as a 'switch' between ceramide and DG-mediated effects or through increased sphingomyelin bisoynthesis with subsequent effects on membrane composition is not clear. Indeed, whether PC-derived DGs are intracellular messengers has been questioned recently [51]. However, the ratio of the lipid second messengers ceramide and DG probably determines the cellular response and given that multiple pathways are involved in regulation of the levels of these lipids, changes in SMS activity are unlikely to always correlate with a single cellular response.

Sphingomyelin degradation

Many stimuli including cytokines, G protein-coupled receptors and stress induce the hydrolysis of sphingomyelin to ceramide and phosphorylcholine, a reaction catalysed by sphingomyelinases. This appears to be the major route for agonist-induced generation of ceramide [52–54] although there is evidence for stimulation of de novo synthesis by agonists also [54]. To date, seven mammalian sphingomyelinases have been identified that vary according to their pH optima, dependence on cofactors, such as magnesium and zinc, and cellular localisation [reviewed in refs. 53, 55]. Two of these activities have been cloned – an acid sphingomyelinase (A-Smase) [56, 57], which is deficient in Niemann-Pick disease, a lysosomal storage disorder [58, 59], and a Mg^{2+}-dependent neutral sphingomyelinase [60]. An in-depth review of sphingomyelinases has been published recently [55]; therefore, only a brief overview will be presented here highlighting the most recent developments. Despite many studies, the individual roles of the different sphingomyelinases in cell signalling remain unclear. For example, early studies implicated a Mg^{2+}-dependent neutral sphingomyelinase (N-Smase) in tumour necrosis factor (TNF), interleukin-1 and ionising radiation responses [61–63], and localisation of an N-Smase to the plasma membrane [64, 65] supported a role for this enzyme in ceramide generation. However, later studies showed that an A-Smase was involved in some TNF actions such as activation of the nuclear transcription factor NFκB [66]. A model to explain these differences was proposed by Kronke and coworkers [67] who modified the cytoplasmic domain of the TNF receptor to show that different receptor domains link to distinct sphingomyelinases. A membrane-proximal region of the cytoplasmic domain linked N-Smase to the mitogen-activated protein kinase (MAPK) cascade, whereas the carboxyl terminus of the TNF receptor connected A-Smase to NFκB activation, demonstrating the ability of a single agonist to activate more than one type of sphingomyelinase and differentially regulate two distinct signalling pathways. The different pH optima and mechanisms of activation suggest that compartmentalisation of sphingomyelinases may be important for generation of ceramide at precise intracellular sites, and a TNF-responsive sphingomyelin pool has been identified at the inner surface of the plasma membrane [35]. The role of A-Smase in agonist-induced responses has remained controversial, because this enzyme is found predominantly in lysosomes [53] and ceramide formed in these compartments is unlikely to have access to downstream effectors. However, A-Smase was recently identified in caveolae [68], suggesting that this enzyme could be involved in generation of ceramide at the plasma membrane. Additionally, the localisation of sphingomyelin predominantly in the outer leaflet of the plasma membrane raises questions as to how intracellular sphingomyelinases might gain access to their substrate. Two recent studies suggest mechanisms by which substrate topography may be overcome (fig. 4). Using a fluorescent sphingomyelin analogue, Tepper et al. [26] have shown that ceramide formed 1–4 h following CD95 stimulation of Jurkat T cells was derived from sphingomyelin originally in the outer leaflet of the plasma membrane. An essential step in the generation of ceramide was the flipping of sphingomyelin to the inner leaflet in a process of lipid scrambling, where it was accessible to hydrolysis by an N-Smase. CD95 stimulation did not increase sphingomyelinase activity, demonstrating that in non-stimulated conditions, separation of

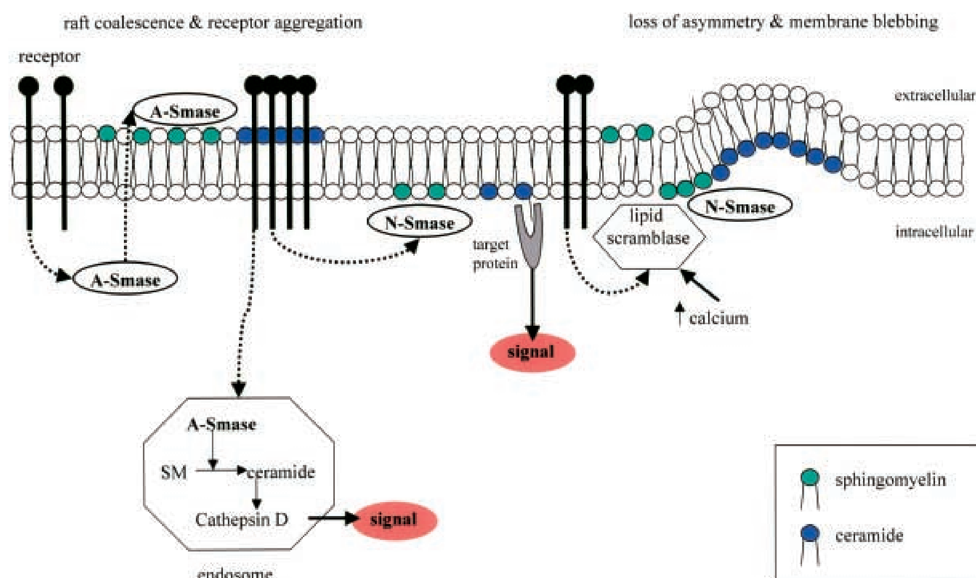

Figure 4. Cellular sites and mechanisms of ceramide action. Receptor activation of A-Smase induces hydrolysis of sphingomyelin in the extracellular leaflet of the plasma membrane. The increase in ceramide facilitates raft coalescence and receptor aggregation [36, 103] and further signalling to endosomal A-Smase where the ceramide generated interacts directly with cathepsin D [95, 96] and cytosolic N-Smase that hydrolyses a pool of sphingomyelin located in the intracellular leaflet of the plasma membrane [35] generating ceramide and recruiting target proteins to the membrane [101]. In addition, receptor activation stimulates lipid scramblase activity which transfers sphingomyelin from the outer to the inner leaflet where it is hydrolysed by N-Smase to ceramide and is involved in the morphological changes associated with apoptosis [26].

sphingomyelin and sphingomyelinase was sufficient to restrict hydrolysis. In a separate study, Grassme et al. [36] showed that CD95 stimulation of lymphocytes induced translocation of A-Smase to the outer surface of the plasma membrane and acute generation of ceramide at the cell surface, within 2 min, and that this step was necessary for receptor clustering and subsequent induction of apoptosis. Whilst these studies demonstrated sphingomyelin hydrolysis at different faces of the plasma membrane, they both showed that the ceramide generated changed the properties of the plasma membrane, facilitating lipid raft coalescence and receptor aggregation in the outer leaflet [36] and apoptotic membrane blebbing/vesiculation when generated in the inner leaflet [26]. These studies present further evidence that different sphingomyelinases are involved in distinct though possibly co-ordinated or interconnected cellular responses.

Ceramide

The observation that sphingolipids could act as bioactive molecules and the discovery of the sphingomyelin cycle, analogous to the PI cycle (sphingomyelinase-mediated hydrolysis of sphingomyelin to produce ceramide and the subsequent replenishment of sphingomyelin levels by sphingomyelin synthase [10]), stimulated interest in the role of sphingomyelin derivatives in cellular responses. Activation of sphingomyelinases occurs in response to a variety of stimuli, including cytokines, antibody receptors, steroids, G protein-coupled receptors and cellular stress [11, 14, 54, 69, 70] and ceramide has been implicated in apoptosis, cellular senescence, growth arrest and differentiation [11, 52, 54, 71]. The ceramide formed during cell stimulation can be further processed to sphingosine and sphingosine 1-phosphate (S1P), both of which are signalling molecules in their own right (see below). Ceramide levels may also be reduced by the action of glucosylceramide and lactosylceramide synthases [11]. Whilst considerable research into sphingomyelinases and the production of ceramide has been undertaken, fewer studies have investigated the regulation of the enzymes involved in its clearance. De novo synthesis of ceramide is also emerging as an important pathway for increasing ceramide levels in response to TNF and chemotherapeutic agents [54]. Ceramide produced by this pathway is delayed, and ceramide synthase appears to be the regulated enzyme [11]. De novo production of ceramide is implicated in cell cycle arrest [72] and apoptosis [52, 73]. Other signalling events in which ceramide has been implicated include activation of MAPKs [74], inhibition of PI3K activation [75–77], regulation of smooth muscle tone [70, 78–80], oxidative stress responses [54, 74] and nitric oxide signalling [11, 81]. The evidence that ceramide is an important second messenger in many cellular functions in response to diverse stimuli has been reviewed extensively [10, 14–16, 52, 69]. Whilst there are conflicting reports concerning the sphingomyelinases involved [55], the time course of ceramide generation [14]

and the role of ceramide in apoptosis [82, 83], overwhelming evidence supports the view of ceramide as a second messenger. However, its mechanisms of action are less clear.

Downstream effectors of ceramide

Treatment of cells with short-chain cell-permeable ceramide has identified many enzymes and signalling molecules as potential targets for this lipid second messenger. The information from such studies must be interpreted with caution, because the high concentrations and prolonged treatments with these synthetic analogues may not truly mimic the time course, site of production or concentration of ceramide produced in response to physiological agonists. Furthermore, the commonly used C_2-ceramide may more closely resemble sphingosine than endogenous long-chain ceramide. Delivery of long-chain endogenous ceramide species to U937 cells induced apoptosis at nanomolar concentrations [84], more closely reflecting the physiological response. Other studies have used inhibitors of ceramide metabolism to increase its intracellular levels, but this may not induce the production of ceramide at a physiologically relevant intracellular site. In addition, many of the reported ceramide responses appear to be indirect and the signalling intermediaries have not been identified. However, several proteins have been identified that interact directly with ceramide. A cytosolic serine/threonine phosphatase, ceramide-activated protein phosphatase (CAPP) [85] was subsequently identified as a member of the 2A class of protein phosphatases (PP2A) [86]. A second serine/threonine phosphatase, PP1, is also emerging as a ceramide-activated phosphatase [87]. Targets for CAPPs include the transcription factor c-Jun (PP2A) [88], the retinoblastoma gene product (Rb) [89] and, more recently, PKCα and Bcl2 [90, 91]. A ceramide-activated protein kinase (CAPK) has also been identified [92] and recently shown to be kinase suppressor of ras (KSR) [93]. In Cos-7 cells, TNF-α or ceramide analogues increased KSR autophosphorylation and its ability to complex with, phosphorylate and activate Raf [93]. Raf is an upstream regulator of the MAPK signalling cascade whose activation is implicated in the inflammatory response to TNF-α. PKCζ [94] and the protease cathepsin D [95] may also be direct targets for ceramide. TNF-α and ceramides activate PKCζ in U937 cells and this may link cytokines to NFκB signalling [94]. Cathepsin D is endosomally active and may link A-Smase-generated ceramide in endosomes to apoptosis [95–97].

Mechanisms of ceramide action

Ceramide is a small hydrophobic neutral lipid that remains within the membrane bilayer in which it is generated. This property was recently demonstrated when ceramide was shown to take days to diffuse from liposomes with a high ceramide concentration to those with low concentration [98]. Therefore, for ceramide to interact directly with downstream effectors they must be present at, or translocate to the site of ceramide generation. Translocation of PKCζ to the particulate fraction following treatment with cell-permeable ceramide has been demonstrated in astrocytes [99].

The structural similarities between ceramide and DG (fig. 2) raise the possibility that these two lipids utilise similar domains for interacting with target proteins. DG binds to proteins through specific lipid-binding domains known as cysteine-rich domains (CRDs) or 'zinc-butterflies'. KSR and PKCζ both possess CRDs, suggesting this may be the site of interaction with ceramide [100]. However, detailed studies with cathepsin D showed that only sphingosine-based lipids but not DG competed for binding [95], indicating the existence of a ceramide-specific binding site. Further studies are required to identify this binding motif. As ceramide cannot move out of the bilayer, it must interact with target proteins from within the bilayer and a possible model has been suggested by Kronke et al. [101]. In this model, one of the side chains of the lipid protrudes from the bilayer and inserts into a hydrophobic pocket in the target protein anchoring it to the membrane (fig. 4). Should such a mechanism occur with ceramide, it is likely to be the alkyl side chain that protrudes from the membrane, because the double bond in this chain is essential for activity [102] and for binding to cathepsin D [95]. Finally, the cellular response to increases in ceramide will be restricted by the compartment in which ceramide is generated, unless specific transport proteins exist to transfer ceramide generated at one intracellular membrane to interact with signalling molecules located at another intracellular site. Clearly, further studies are required to identify intracellular sites of ceramide production and to colocalise specific target proteins at these sites to better understand the role of this lipid second messenger in complex signalling pathways.

Ceramide in apoptosis and cellular senescence

The majority of evidence suggests that ceramide is an important mediator of apoptosis [reviewed in refs. 16, 52, 74]. The precise mechanisms of ceramide action are still unresolved but appear to fall into three main categories: (i) acute transient activation of A-Smase increases ceramide at the extracellular face of the plasma membrane promoting receptor aggregation and subsequent signalling [36, 103]; (ii) sustained ceramide production by either early caspase-dependent N-Smase activation or by de novo synthesis activates CAPPs leading to late caspase activation and apoptosis [reviewed in ref. 74] and (iii) plasma membrane phospholipid scrambling occurs pre-

senting sphingomyelin to N-Smase at the cytosolic face and the production of ceramide which is necessary for membrane blebbing and morphological changes associated with apoptosis (fig. 4) [26]. CAPPs also appear to be involved in cellular senescence. In molt-4 leukaemia cells, ceramide causes G0/G1 cell-cycle arrest [14, 71]. This effect is mediated through ceramide activation of CAPPs and dephosphorylation of Rb. Mechanistic studies have shown how these two effects of CAPPs, activation of caspases and apoptosis and dephosphorylation of Rb and growth arrest, may be regulated. Bcl2 overexpression [104] or inhibition of PKC [14] prevents ceramide-induced caspase activation but not Rb dephosphorylation. Conversely, when Rb was absent or rendered inactive, ceramide no longer induced cell cycle arrest [14]. Therefore, the outcome – apoptosis or growth arrest – of an increase in ceramide will depend on the downstream effectors and their regulators present in the cells under study [74].

Additional signalling pathways affected by ceramide

Cross-talk between ceramide and glycerophospholipid signalling may also occur with the potential to determine between the cell growth and cell death pathways. For example, ceramide inhibits PLD activity blocking the mitogenic PKC pathway [4, 105]. Another mechanism by which ceramide may determine whether cell survival or death occurs in response to certain stimuli is through cross-talk with the PI3K pathway. Stimulation of this pathway leads to sequential activation of PI3K, PDK and Akt/PKB, which inhibits apoptosis through phosphorylation of Bcl2 [8]. C2-ceramide inhibits this pathway either by downregulating PI3K activity [76, 106] or by preventing Akt/PKB activation [107–109] so inhibiting cell survival and promoting apopotosis. In contrast, stimulation of PI3K activity by C2- and C6-ceramide has been reported. In smooth muscle cells, C2-ceramide-induced activation of PI3K occurs through a tyrosine kinase-dependent mechanism and is involved in contraction [80, 110]. Gulbins et al. [111] proposed that ceramide ativation of PI3K was necessary for apoptosis, whereas Hanna et al. [75] invoked PI3K activation by ceramide in fibroblast proliferation. Finally, activation of PI3K has recently been shown to inhibit TNF-α-induced apoptosis by reducing ceramide production [77], and expression of constitutively activated Akt/PKB inhibited ceramide-induced apoptosis [112]. Therefore, the interactions of the ceramide and PI3K pathways appear complex, and the cellular outcome of their dual activation will undoubtedly reflect the signalling components expressed and the background in which they are activated.

Sphingosine and S1P

Cell signalling functions have been ascribed to both sphingosine and S1P. Sphingosine has been shown to inhibit PKC [13] and to stimulate cell growth and differentiation [11, 113]. S1P is implicated in migration, differentiation, mitogenesis and apoptosis [11, 113, 114]. Sphingosine is formed by deacylation of ceramide catalysed by ceramidases (fig. 5) and because there is no evidence for de novo synthesis of sphingosine in mammalian cells, ceramidases may be important regulators of ceramide and sphingosine levels during agonist stimulation. Three main forms of ceramidase activity have been identified: acid, neutral and alkaline [11]. Acidic and neutral ceramidases can be activated by cytokines [115], whereas alkaline ceramidase is stimulated by growth factors but not cytokines [116].

Sphingosine kinase

Ascribing a specific cellular function to sphingosine is difficult as it can be rapidly phosphorylated by sphingosine kinase (SPHK) to S1P (fig. 5). The two mammalian SPHKs cloned show a broad tissue distribution at the mRNA level, being most abundant in lung and spleen [117]. Cloned murine SPHK has at least three calcium/calmodulin-binding sites as well as phosphorylation sites for PKA, casein kinase II and PKC [114]. The enzymes, which are found in both the cytosol and membrane compartments [118, 119], are activated by phorbol esters (PKC activators), suggesting that PKC phosphorylation regulates their activity. Certainly, there is evidence that PKC activation, which is accompanied by increased SPHK activity and S1P formation, inhibits ceramide-induced apoptosis [74, 120]. They can also be regulated by acidic phospholipids such as phosphatidic acid and phosphatidylserine, suggesting an interaction with glycerophospholipid signalling.

S1P degradation

S1P levels are regulated by S1P lyase and S1P phosphatase. S1P lyase cleaves S1P at the C2-C3 bond to produce palmitaldehyde and phosphoethanolamine [121]. A mammalian S1P lyase has been cloned [122] and shown to have a transmembrane domain localising the enzyme to the membrane. S1P lyase mRNA was most abundant in liver, kidney, lung and brain [122]. Additionally, S1P may also be dephosphorylated back to sphingosine, a reaction catalysed by non-specific lipid phosphatases [114], although in mammalian cells, a S1P phosphatase activity distinct from non-specific lipid phosphatase activity has been detected [121].

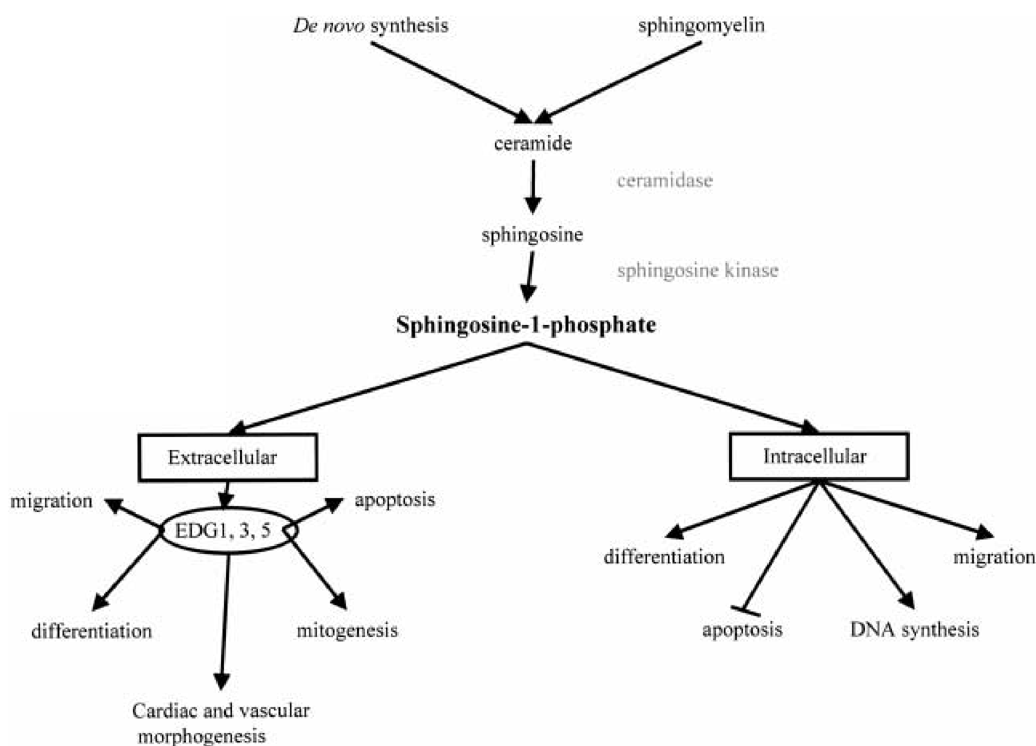

Figure 5. Signalling by sphingosine-1-phosphate. Sphingosine-1-phosphate is generated from ceramide by the sequential action of ceramidases and sphingosine kinases. The activity of both of these enzymes can be regulated by extracellular stimuli such as cytokines and growth factors [114]. Sphingosine-1-phosphate can signal by two mechanisms: (i) intracellular – as a second messenger interacting with many signalling cascades, and (ii) extracellular – through specific receptors belonging to the endothelial-differentiating gene family. The major cellular responses in which sphingosine-1-phosphate has been implicated are shown.

Sphingosine-1-phosphate

SPHK activity and S1P levels increase in response to stimuli such as growth factors, G protein-coupled receptors, cytokines, phorbol esters, vitamin D3 and antigen. Both transient acute accumulation of S1P and chronic elevation have been observed [reviewed in ref. 114]. The mechanisms regulating SPHK activity are not yet defined. Calcium mobilisation and tyrosine kinase activity have been implicated in platelet-derived growth factor responses [123] but SPHK activation appears to precede calcium elevation in antigen-stimulated mast cells [124]. PLD and G proteins are also implicated in SPHK activation [125, 126].

Signalling by S1P

S1P is unique in the sphingolipid signalling system in its ability to act as an extracellular stimulus through specific cell surface receptors and as an intracellular second messenger through direct activation of signalling proteins (fig. 5). Extracellular S1P signals through G protein coupled receptors belonging to the endothelial-differentiating gene (EDG) family. It has high affinity for EDG1, EDG3 and EDG5, which are important for endothelial cell tubule formation and angiogenesis [127]. EDG receptors couple to Gi, Gq and G12/13 heterotrimeric G protein family members and regulate signalling through MAPK, PKC and Rho pathways. Their activation by S1P has been implicated in diverse cellular responses including differentiation, migration, mitogenesis and apoptosis [reviewed in refs 11, 114]. S1P has also been proposed to play a second messenger role, following the observation that sphingosine-induced calcium release was dependent on SPHK activation [128, 129]. However, distinguishing between the intracellular effects of S1P and its EDG receptor-mediated responses is difficult, particularly as more than one EDG receptor type coupling to different G proteins and downstream effectors may be expressed in individual cells. Furthermore, the responses attributed to intracellular S1P, such as activation of MAPKs, PLD and p125FAK, and calcium release resulting in regulation of DNA synthesis, apoptosis, differentiation and migration are similar to the effects of EDG receptor activation. For references and evidence of the role of S1P in signalling, readers are referred to a recent comprehensive review [114].

S1P in vascular and cardiac morphogenesis

Two recent studies have shown that EDG receptors are important for embryonic formation of the heart and blood

vessels. The role of EDG receptor signalling appears to be regulation of smooth muscle cell migration in the embryo. The heart originates from two patches of tissue (primordia) located on either side of the vertical axis that marks the embryo midline. During development, the heart primordia move towards the midline and merge forming the heart [130]. Kupperman et al. [131] have shown that a gene, called 'miles apart', is essential for guiding the migration of the primordia during the initial formation of the heart tube in zebrafish development. They also showed that miles apart encoded a lysosphingolipid-binding G protein-coupled receptor – mil – and that S1P was most probably its ligand. In the second study, Liu et al. [132] investigated the role of EDG1 receptor in development using an the EDG1$^{-/-}$ mouse. The EDG1 knockout resulted in embryonic lethality due to haemorrhage. This was due to incomplete vascular maturity, such that smooth muscle cells did not migrate to fully surround the endothelial lumen of the blood vessels. These two studies are the first to show that a sphingolipid signalling system controls cell migration in vertebrate embryos. In addition, both studies demonstrated that the S1P receptor did not have to be expressed by the migrating cells, suggesting that the effects of S1P are mediated through release of chemoattractants or a critical adhesion factor by the remote cells. Given that smooth muscle cell migration goes awry in many types of vascular disease, for example atherosclerosis, these studies suggest that modulation of S1P receptor signalling may be an important therapeutic target. However, the mechanisms underlying the effects of S1P need to be clarified by further study.

Interactions with cholesterol and proteins

Sphingolipid-enriched membrane microdomains

Recent studies in intracellular signalling, particularly in haematopoietic cells, have established the importance of specialised membrane domains that act as centres for co-localisation or recruitment of receptors and signalling molecules. These domains are called lipid rafts or detergent-insoluble glycolipid-enriched domains due to their resistance to solubilisation by non-ionic detergents at 4 °C [for a recent review see ref. 133]. In certain cell types, for example endothelial and smooth muscle cells, these lipid domains are associated with caveolae, flask-like invaginations of the plasma membrane, which again appear to function, at least in part, as centres for cell signalling [134, 135]. Studies of the physical characteristics of different classes of lipids and their behaviour in model membranes suggest a biophysical basis for the formation of these domains. Sphingomyelin and PC compose approximately 50 % of the phospholipids present in the outer leaflet of the plasma membrane in mammalian cells [33]. The relatively long, predominantly saturated acyl side chains of sphingolipids give them a cylindrical shape and allow tight packing; in contrast, glycerophospholipids with shorter and less saturated 'kinked' acyl chains pack less tightly. In addition, sphingolipids have a higher melting temperature than glycerolipids and these different physical properties allow for phase separation and the formation of 'rafts' of rigid tightly packed sphingolipids floating in more fluid glycerolipid-enriched domains [17]. Originally, bilayer lipids were envisaged to form a fluid environment allowing diffusion of membrane proteins in the plane of the membrane [136]. However, it is now clear that lipids are more structured within membranes and an updated version of the original, 'fluid mosaic' membrane model has been proposed [17, 137]. In this revised model, phospholipids are arranged asymmetrically between the bilayers (described above). Additionally, there is lateral heterogeneity with the existence of structured domains and microdomains in the plane of the membrane. However, to understand the formation of lipid rafts in biological membranes, the effects of another membrane constituent sterols (in mammalian cells, cholesterol), must be taken into account. Although mainly found in the plasma membrane, cholesterol is also present in most intracellular membranes [138], while in model membranes, it promotes phase separation of lipids with different melting temperatures [139, 140]. This suggests that in cell membranes, cholesterol would act to promote the formation of sphingolipid-enriched domains. Studies of the interactions of phospholipids with cholesterol in model membranes suggest that although cholesterol is not essential for the formation of sphingolipid domains, its presence alters their physical characteristics significantly. In the absence of cholesterol, the sphingolipid domains would be rigid and allow little diffusion of molecules within, into or out of the domains [31]. In the presence of cholesterol (>25 mol%), the sphingolipids would remain tightly packed but the domain would be less rigid due to cholesterol molecules acting as spacers and forming a 'lattice' structure in which the diffusion of molecules such as proteins could be regulated [for references and detailed recent reviews of the interactions of sphingomyelin and cholesterol see refs 29, 141, 142]. Certainly, there is evidence for such interactions in biological membranes, because lipid rafts are enriched with cholesterol and depletion of cholesterol from cell membranes disrupts these domains [143]. Finally, in addition to acting as a 'spacer' and stabiliser of lipid rafts, cholesterol may also affect their size and shape by localising along the junctions between lipid phases, so forming a boundary [144].

Signalling through lipid rafts

Lipid rafts are emerging as important centres for cell signalling. Many different types of signalling molecules as-

sociate with rafts, including proteins that interact at the outer and inner leaflet of the plasma membrane as well as transmembrane proteins and lipids such as PIP_2 and glycosphingolipids (e.g. gangliosides). The majority of lipid raft-associated proteins are themselves lipid modified which would favour their interaction with the ordered characteristics of lipid rafts. Examples of these modifications include (i) a glycosylinositolphosphate (GPI) anchor, which inserts into the outer plasma membrane leaflet, e.g. IgG receptors, (ii) palmitoylation or myristoylation, in which the acyl chain inserts into the cytoplasmic face of the plasma membrane, e.g. Src family tyrosine kinases and (iii) farnesylation, which induces a labile association with the inner leaflet, e.g. Ras [reviewed in refs 18, 134, 135, 141]. Additional signalling molecules reported to be associated with lipid rafts and or caveolae include growth factor receptors, G protein-coupled receptors, extracellular regulated kinases (ERK1 and 2), endothelial nitric oxide synthase, PKC, EDG1 and integrins [133, 135, 145]. Many cell surface receptors, including T-cell receptors [18], TNF receptor [146], integrins [147] or growth factors [148] aggregate upon activation, migrate towards one pole of the cell and coalesce into 'caps' [148, 149]. 'Capping' is thought to facilitate signal transduction by assembling various components of the signalling cascade. Disruption of lipid rafts by cholesterol depletion prevents clustering of many receptors, including T cell receptor [18], CD48 [150], Fcγ [151] and TNF receptor [152]. In addition, cholesterol depletion prevented receptor tyrosine phosphorylation and signalling in T cells [153]. Recently, Grassme et al. [36] have shown that sphingomyelin hydrolysis and the generation of ceramide at the extracellular face of the plasma membrane is required for CD95 receptor clustering in Jurkat cells. Disruption of lipid rafts with cholesterol prevented ceramide generation and receptor aggregation. In a separate study, Cremesti et al. [103] showed that intact lipid rafts and ceramide generation were essential for capping of Fas and induction of apoptosis following anti-Fas antibody treatment. These studies and others [reviewed in refs 18, 141] suggest an important function for lipid rafts in receptor aggregation and subsequent signalling.

Sphingolipid signalling in caveolae

Caveolae are flask-like invaginations of the plasma membrane enriched with caveolin and cholesterol, and they appear to act as centres for signal transduction [17, 135, 154]. Lipid rafts and caveolae membranes share many characteristics: both are enriched with sphingolipids and cholesterol, and GPI-anchored proteins and Src tyrosine kinases associate with both types of membrane domain. In addition, both are buoyant detergent-resistant plasma membrane domains that separate to similar fractions on sucrose density gradients [141, 155]. However, recent evidence suggests that these domains exist as separate entities within the plasma membrane. Using antibodies against gangliosides GM1 and GM3 – glycosphingolipids enriched in detergent-resistant membranes – GM1 has been localised to caveolae [156] and GM3 to lipid rafts [157, 158]. Whilst the lipid composition of lipid rafts has been studied extensively [reviewed in ref. 141], less is known of the lipids present in caveolae membranes. High levels of cholesterol and low levels of sphingomyelin were found in caveolae membranes compared to lipid rafts in mouse melanoma B cells [157]. In NIH3T3 cells, caveolae membranes were enriched with sphingomyelin and neutral glycosphingolipids in addition to cholesterol [159]. Pitto et al. [160] using a photoactivable radiolabelled derivative of GM1 that inserts into caveolae membranes and cross-links lipids and proteins when light activated [161] showed that sphingomyelin and ganglioside are enriched on the outer leaflet of the caveolar bilayer. The function of sphingolipids in caveolae is not clear. Depletion of neutral glycosphingolipids had no effect on caveolar morphology in NIH3T3 cells [159] suggesting that this class of sphingolipid did not have a structural role. However, cholesterol depletion did disrupt caveolae in smooth muscle cells [162] and as in lipid raft preparations affected signal transduction. For example, cholesterol depletion inhibits bradykinin-stimulated PI turnover, probably by decompartmentalisation of PIP_2 from lipid rafts [19], and causes hyperactivation of p42MAPK in response to epidermal growth factor [163]. Caveolae have also been implicated in regulation of vascular tone because their disruption by cholesterol depletion decreased the production of calcium sparks in smooth muscle cells [162]. This observation is potentially important because calcium sparks are involved in vasorelaxation through activation of calcium-activated potassium channels, which hyperpolarize the membrane [164]. The β1 subunit of the calcium activated potassium channel confers calcium sensitivity, and β1-knockout mice were recently shown to be hypertensive [165]. These studies suggest that sphingolipids have a structural role in caveolae function. There is also evidence that sphingomyelin hydrolysis and ceramide generation may occur within caveolae. An A-Smase activity appears to be present in caveolae and 70–50% of cellular ceramide is found in these structures [68, 166]. Interleukin-1β and low-affinity neurotrophin receptor (p75NTR) have both been shown to induce sphingomyelin hydrolysis in caveolae [68, 167]. Recently, a neutral sphingomyelinase activity has been found in caveolae isolated from human fibroblasts [168]. The activity of this enzyme appeared to be inhibited when associated with caveolae, possibly through binding to caveolin. However, its role in signalling is unclear, because although TNF-α treatment led to recruitment of TNF receptors to caveolae, the resultant sphingomyelin hydroly-

sis and ceramide formation occurred at sites other than caveolae [168]. Three possible roles of sphingomyelin hydrolysis and ceramide production in caveolae have been suggested: (i) it may be necessary for p75NTR- and TNF-α-induced apoptosis [53, 169] although this is not supported by a recent study showing no production of ceramide in caveolae in response to TNF-α in skin fibroblasts [168], (ii) cross-talk with tyrosine kinase signalling may occur [166] and (iii) increases in ceramide within caveolae may alter membrane properties by favouring greater curvature of the membrane [102]. The latter possibility is supported by studies in model membranes where treatment with bacterial sphingomyelinase induced the formation of ceramide-enriched microdomains [170]. However, a final caveat to be borne in mind is whether sphingolipid-enriched domains are actually present within caveolae or whether caveolae-associated membranes are present in the caveolae preparations as a consequence of the techniques used to prepare them [166]. Further studies examining the lipids present in caveolae membranes are required before the signalling role of sphingolipids in this membrane domain can be fully appreciated.

Summary

In this review we have summarised the recent advances in our knowledge of the role of sphingolipids in mammalian cell signalling. In particular, we have tried to draw together the classical second messenger roles of ceramide, sphingosine and S1P with the structural roles of glycosphingolipids, sphingomyelin and ceramide. The emerging picture is one of a complex signalling system involved in regulating cellular processes from embryonic development to cell senescence and death. Clearly ceramide is now well established as a second messenger in apoptosis and differentiation, particularly in response to cytokines and stress. However, the mechanisms of ceramide action are less clear, as this lipid appears to act directly through interactions with target proteins and indirectly by altering membrane properties, so facilitating receptor aggregation and morphological changes associated with apoptosis. Compartmentalisation of sphingomyelinases and sphingomyelin at separate locations within the cell and the existence of complex mechanisms to maintain this topography ensures that the generation and site of action of ceramide is regulated. The observation that signalling molecules associate with sphingolipid-enriched membrane domains has sparked intense interest in the function of such ‘rafts’ in cell signalling. However, whether sphingomyelin hydrolysis and ceramide generation occur in rafts is still unclear and further studies are required before there can be a full understanding of the function of these sphingolipid-enriched structures. An important new development is the identification of the S1P receptor EDG as a regulator of embryonic vascular and cardiac morphogenesis. This demonstrates that sphingolipid signalling pathways are involved in cardiovascular development and future studies will undoubtedly cast light on the pathophysiological role of S1P in processes such as angiogenesis and atherosclerosis.

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