

Spingosine and Ceramide Signalling in Apoptosis

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Spingosine and ceramide belong to a structurally diverse group of lipids, collectively known as sphingolipids, that are abundant in membranes and are gaining recognition as important signalling mediators (reviewed in 1-4). Spingosine and ceramide are prototypic sphingolipids, in that they are composed of a hydrophobic sphingoid long chain base (**Fig. 1**). Ceramide also has a variable length fatty acid chain attached by an amide bond at the carbon 2 position (**Fig. 1**). Ceramide represents the basic building block of more complex sphingolipids, such as plasma membrane sphingomyelin, and is either synthesised *de novo* or generated from sphingomyelin breakdown (**Fig. 1**). In contrast, spingosine is solely produced by de-acylation of ceramide and can also be converted back to ceramide by a scavenger pathway or converted to spingosine-1-phosphate by spingosine kinase (**Fig. 1**), and is the topic of the fourth article in this Showcase on Research. The inter-convertibility of spingosine and ceramide has meant that interpreting their effects and distinguishing their individual roles *in vivo* has proven problematic. However, studies employing *in vitro*

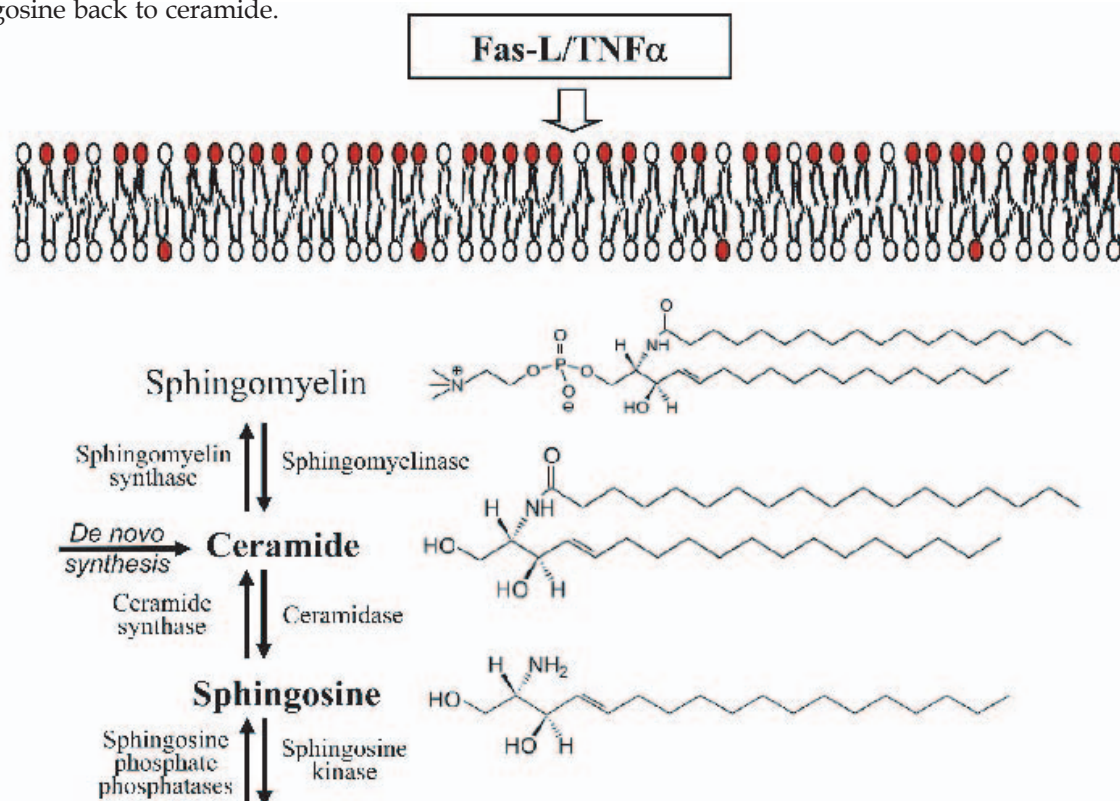
approaches are beginning to reveal individual roles for these lipids in regulating kinases and proteins involved in key signalling events that may explain their biological effects. This review aims to provide an overview of spingosine and ceramide biology and to reconcile *in vivo* biological activities with their demonstrated effects on key signalling molecules.

Ceramide and Spingosine Accumulate in Apoptotic Cells

In healthy cells, sphingomyelin is found predominantly in the outer leaflet of the plasma membrane (**Fig. 1**). However under conditions of stress, sphingomyelin turnover is induced resulting in its breakdown and an increase in ceramide and spingosine levels (**Fig. 1**). In particular, apoptotic inducers such as tumor necrosis factor α (TNF α) and Fas ligand (Fas-L) cause a rapid and significant accumulation of both sphingolipids in the first hour of treatment; this has been observed in many cell types including human neutrophils, Jurkat T-cells, U937 and HL60 cells. Other agents that induce cell death such as

Fig. 1. The biosynthesis of ceramide and spingosine.

Lipids with red head groups represent sphingomyelin, which is asymmetrically distributed across the plasma membrane in cells. Upon stimulation with apoptotic stimuli such as Fas ligand (Fas-L) or tumour necrosis factor α (TNF α) sphingomyelin is degraded by sphingomyelinase and ceramidase resulting in ceramide and spingosine accumulation. Ceramide is also generated *de novo* by ceramide synthase which also converts spingosine back to ceramide.



anti-cancer agents and environmental stress similarly induce ceramide and sphingosine accumulation (Table 1). This common theme has led to the hypothesis that these lipids may contribute to the apoptotic fate of the cell. Furthermore, both sphingosine and ceramide have been shown to induce apoptosis in many cell types including cancer cells, and this has created interest in their use as potential anti-cancer agents (5, 6). However, the underlying molecular mechanisms involved in sphingolipid-induced apoptosis are still poorly understood. Several key observations have been made in relation to changes in signalling pathways induced by sphingosine and ceramide consistent with well-described apoptotic mechanisms.

Table 1. Inducers of cellular ceramide and sphingosine accumulation.

Apoptotic stimuli	Environmental stresses	Chemotherapeutic agents
Fas TNF α Interleukin-1	UV radiation Growth factor withdrawal Serum deprivation	Etoposide Doxorubicin Adriamycin

Ceramide and Sphingosine Induce Changes in Cells Consistent with Apoptosis

Treatment of cells with either sphingosine or ceramide has been demonstrated to inhibit the activity of the mitogen-activated protein kinases (MAPKs), Erk-1 and Erk-2 and simultaneously activate stress-activated protein kinases (SAPKs) such as JNK and p38 in a variety of cell types (3, 4). This same imbalance of MAPKs and SAPKs is also associated with apoptosis induced by growth factor withdrawal (7). The time course of sphingosine-induced MAPK inhibition and SAPK activation is consistent with the time course of sphingosine accumulation, but *in vitro* studies have demonstrated that the effect of sphingosine on MAPK activation is not direct (8). This suggests that sphingosine acts upstream of Erks in inactivating the MAPK pathway.

Sphingosine and ceramide have been shown to induce apoptosis in cells by the mitochondrial pathway. In particular, down-regulation of the PI3K-Akt pathway is associated with sphingosine and ceramide treatment of cells, leading to dephosphorylation of Bad and activation of apoptotic pathways at the mitochondria. Over-expression of active Akt partially counteracts the apoptotic activity of sphingosine and ceramide, suggesting that disruption of this pathway contributes to the apoptotic activity of these lipids (4). Additionally, over-expression of Bcl-2 provides a protective effect against ceramide- and sphingosine-induced cell death by negating the effects of the sphingolipids on mitochondrial depolarisation, a critical step in the induction of apoptosis. Thus sphingosine and ceramide exert their apoptotic effects upstream of the

mitochondrial control of apoptosis.

The apoptotic activity of sphingosine and ceramide on cells has generated interest in the underlying molecular mechanisms involved. The effectors of ceramide and sphingosine signalling are gradually being uncovered and those identified to date are presented in the following sections. Ceramide and sphingosine are discussed separately as despite their overlapping biological effects and inter-convertibility, most of the mechanisms and targets identified have been studied in relation to one or other of these sphingolipids. In the following discussion examples are limited to cases where a direct lipid effect has been demonstrated both *in vitro* and *in vivo*.

Ceramide Exhibits Diverse Effects on Signalling Complexes

The turnover of sphingomyelin at the plasma membrane results in the accumulation of ceramide in sphingolipid-rich domains known as lipid rafts (1, 3, 9). The biophysical characteristics of ceramide drive the coalescence of lipid rafts into larger platforms in which signalling events are potentiated due to clustering of receptors and signalling molecules (1, 9). In this way ceramide

accumulation influences apoptotic signalling by membrane receptors. Recent evidence also suggests that ceramide-rich rafts formed in response to UV radiation contribute to apoptosis in the absence of membrane receptor signalling (10). Ceramide also accumulates in mitochondrial membranes in response to chemotherapeutic drugs and TNF α (1). This causes depolarisation of mitochondria with consequent release of apoptotic mediators from the outer mitochondrial membrane, possibly by ceramide-mediated pore formation (11). Thus ceramide plays a role in apoptotic signalling by altering the biophysical characteristics of membranes.

Direct effectors of ceramide have also been identified and include the kinase suppressor of Raf-1 (KSR-1), originally described as ceramide-activated protein kinase. Ceramide enhances the auto-phosphorylation of KSR-1 and increases its ability to activate Raf-1, resulting in up-regulation of the downstream MAPK pathway (12) (Fig. 2). This effect of ceramide does not correspond to its observed role in apoptosis and suggests that ceramide may have distinct roles apart from inducing apoptosis in cells. In fact ceramide has been shown in some cases to have differential effects, causing either stimulation or inhibition of PKC γ depending upon its concentration (13), suggesting that changes in ceramide levels can act as a biological switch. More recently ceramide has been shown to activate a mixed lineage kinase (MLK) that lies upstream of the SAPK pathway. Ceramide activates this kinase both *in vitro* and *in vivo* and has been shown to mediate TNF α 's effect on JNK (Fig. 2) (14). Therefore ceramide accumulation plays a direct role in apoptosis by regulating the SAPK pathway.

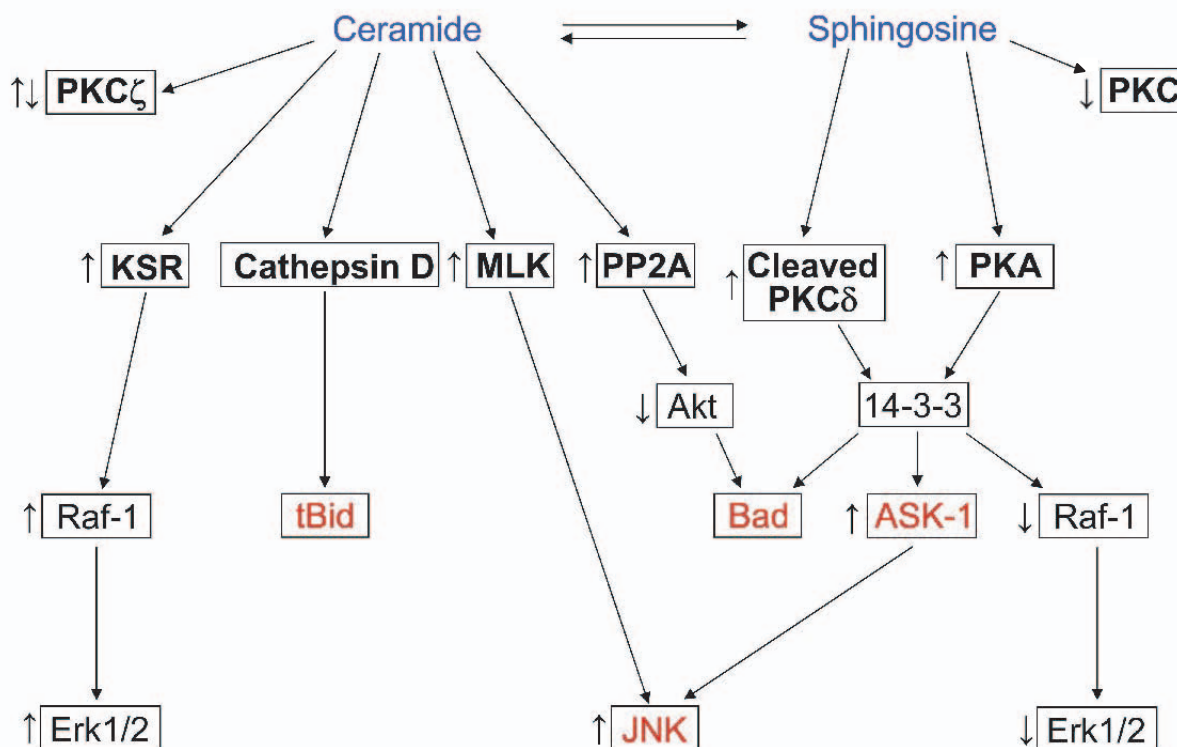


Fig. 2. Ceramide and sphingosine directly affect many signalling pathways. Direct effectors of ceramide and sphingosine are shown in bold. ↑ indicates activation and ↓ indicates inhibition of enzyme activity. Apoptotic mediators are shown in red.

Ceramide also binds directly to the aspartic protease, cathepsin D, which is implicated in TNF α -, Fas- and chemotherapy-induced apoptosis (2). Interaction of cathepsin D with ceramide induces autolytic cleavage of the pro-enzyme, releasing the active components of cathepsin activity. This ceramide-induced cathepsin D activation has recently been shown to mediate cleavage and subsequent activation of the pro-apoptotic Bcl-2 family member Bid (Fig. 2) in response to TNF α (15). Ceramide also directly recruits and activates phosphatase activities (2,3), and via this mechanism inactivates Akt leading to induction of pro-apoptotic Bad (Fig. 2). Hence ceramide accumulation mediates mitochondrial depolarisation by regulating Bcl-2 family members via multiple mechanisms.

In the light of a large and increasing body of evidence, the role of ceramide as a second messenger in apoptotic signalling is now becoming accepted. On the other hand, sphingosine's role in apoptosis is less well established. However recent developments are now beginning to reveal a specific role for sphingosine in apoptosis.

Sphingosine has Direct Effects on Signalling Kinases

One of the first targets of sphingolipid-mediated signalling identified was protein kinase C (PKC) (16). Sphingosine has been demonstrated to be a potent inhibitor of PKC (Fig. 2), so much so that for many years all the apoptotic effects of sphingosine were attributed to PKC inhibition. However, sphingosine has recently been shown to affect other signalling kinases: for example, sphingosine inhibits calmodulin-

dependent kinases (4), yet it enhances signalling by the epidermal-growth factor receptor (EGFR) (17), and activates other kinases including casein kinase II (18), p21-activated kinase 1 (19) and 3-phosphoinositide-dependent kinase 1 (20). Thus sphingosine can positively as well as negatively affect kinase activities and unlike ceramide, is not exclusively confined to membrane fractions, making it an ideal second messenger candidate.

As the complexity of sphingosine's effects on signalling has become apparent, some investigators have taken more global approaches to identify effectors involved. Two groups characterised kinase activities that are directly activated by sphingosine in Jurkat (21) and Balb/3T3 cell extracts (22). In the latter study a kinase activity was isolated that specifically phosphorylated members of the 14-3-3 protein family in response to sphingosine and this was denoted sphingosine-dependent kinase 1 (SDK1). The 14-3-3 proteins are a highly conserved family of ubiquitously expressed dimeric phospho-serine binding proteins that interact and modulate the functions of key cellular proteins involved in signalling (23). SDK1 phosphorylates a site at the interface of the dimeric 14-3-3 molecule and we have subsequently demonstrated that this phosphorylation results in the disruption of the dimeric structure of the protein (24). This finding has profound implications, as dimeric 14-3-3 has been shown to protect cells from apoptosis by controlling pro-apoptotic mediators such as Bad, and the SAPK-activating kinase apoptosis signal-regulating kinase (ASK-1), whilst supporting survival and proliferative functions such as enabling the efficient activation of

Raf-1 (23, 25). Thus sphingosine-induced monomerisation of 14-3-3 protein may mediate the apoptotic fate of cells via disruption of 14-3-3's anti-apoptotic function. This hypothesis is entirely consistent with the observed effect of sphingosine on cells (Fig. 2). Subsequent identification of SDK1 as the caspase-cleaved fragment of PKC δ (26) further emphasises the potential role of this pathway in sphingosine-induced apoptosis (Fig. 2). PKC δ has an established role in apoptosis induced by many DNA damaging agents including chemotherapeutic agents and UV radiation (27). Thus the sphingosine regulation of dimeric 14-3-3 protein function may be a vital clue to the molecular mechanism underlying sphingosine-induced cell death.

In the course of our studies investigating sphingosine-induced phosphorylation of 14-3-3, we have identified another SDK capable of phosphorylating and monomerising 14-3-3. We have shown that protein kinase A (PKA), a kinase otherwise known as cAMP-dependent kinase, is also activatable by sphingosine (28). The localisation of PKA to the plasma membrane via A-kinase anchoring proteins puts it in an appropriate position to be a front-line effector of sphingosine signalling. Indeed we have demonstrated that membrane-associated PKA is sensitive to sphingosine levels *in vivo* (28) indicating that PKA could be a primary effector of sphingosine-induced signalling (Fig. 2). This discovery reveals that sphingosine regulation of dimeric 14-3-3 can be mediated by more than one kinase (Fig. 2), and importantly, PKA, a well studied enzyme, has hitherto unknown biological activities in response to sphingosine.

Future Perspectives

Many cancers exhibit resistance to chemotherapy or radiotherapy and are therefore refractory to conventional treatment. However, drug-resistant cancer cells have been shown to be as sensitive to the apoptotic effects of sphingosine and ceramide as non-drug resistant lines. In some cases the basis of drug-resistance has been shown to be altered sphingolipid metabolism, indicating that the evasion of the apoptotic effects of endogenous sphingosine and ceramide is a mechanism of cancer progression. Currently, sphingosine and ceramide derivatives and agents that modify their endogenous levels are being assessed as potential anti-cancer agents (5, 6). However, a full understanding of the molecular mechanisms underlying the biological activity of these lipids may identify new targets for therapeutic development and lead to drugs with greater specificity and efficacy.

References

1. □ Ohanian, J., and Ohanian, V. (2001) *Cell. Mol. Life Science* **58**, 2053-2068
2. □ Pettus, B.J., Chalfant, C.E., and Hannun, Y.A. (2002) *Biochim. Biophys. Acta* **1585**, 114-125
3. □ Ruvolo, P.P. (2003) *Pharm. Res.* **47**, 383-392
4. □ Olivier, C. (2002) *Biochim. Biophys. Acta* **1585**, 153-162
5. □ Kester, M., and Kolesnick, R. (2003) *Pharm. Res.* **47**, 365-371
6. □ Reynolds, C.P., Maurer, B.J., and Kolesnick, R.N. (2004) *Cancer Letts.* **206**, 169-180
7. □ Xia, Z., Dickens, M., Raineaud, J., Davis, R.J., and Greenberg, M.E. (1995) *Science* **270**, 1326-1331
8. □ Sakakura, C., Sweeney, E.A., and Shirahama, T. (1997) *Int. J. Oncol.* **11**, 31-39
9. □ Dobrowsky, R.T. (2000) *Cell. Signalling* **12**, 81-90
10. □ Rotolo, J.A., Zhang, J., Donepudi, M., Lee, H., Fuks, Z., and Kolenick, R. (2005) *J. Biol. Chem.* **280**, 26425-26434
11. □ Siskind, L.J. (2005) *J. Bioenerg. Biomembr.* **37**, 143-153
12. □ Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X-H., Basu, S. McGinley, M., Chan-Hui, P-Y. Lichenstein, H., and Kolesnick, R. (1997) *Cell* **89**, 63-72
13. □ Muller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D., and Pfizenmaier, K. (1995) *EMBO J.* **14**, 1961-1969
14. □ Sathyanarayana, P. Barthwal, M.K., Kundu, C.N., Lane, M.E., Bergmann, A., Tzivion, G., and Rana, A. (2002) *EMBO J.* **10**, 1527-1533
15. □ Heinrich, M., Neumeyer, J., Jakob, M. Halla, C., Tchikov, V., Winoto-Morbach, S., Wickel, M. Schneider-Brachert, W. Trauzold, A., Hethke, A., and Schutze, S. (2004) *Cell Death Differ.* **11**, 550-563
16. □ Smith, E.R., Merrill, A.H., Obeid, L., and Hannun, Y.A. (2000) *Methods Enz.* **312**, 361-373
17. □ Davis, R.J., Girones, N., and Fauchner, M. (1988) *J. Biol. Chem.* **263**, 5373-5379
18. □ McDonald, O.B., Hannun, Y.A., Reynolds, C.H., and Sahyoun, N. (1991) *J. Biol. Chem.* **266**, 21773-21776
19. □ Bokoch, G.M., Reilly, A.M., Daniels, R.H., King, C.C., Olivera, A., Spiegel, S., and Knaus, U.G. (1998) *J. Biol. Chem.* **273**, 8137-8144
20. □ King, C.C., Zenke, F.T., Dawson, P.E., Dutil, E.M., Newton, A.C., Hemmings, B.A., and Bokoch, G.M. (2000) *J. Biol. Chem.* **275**, 18108-18113
21. □ Pushkareva, M.Y., Khan, W.A., Alessenko, A.V., Sahyoun, N., and Hannun Y.A. (1992) *J. Biol. Chem.* **267**, 15246-15251
22. □ Megidish, T. Cooper, J. Zhang, L. Fu, H., and Hakomori, S.I. (1998) *J. Biol. Chem.* **273**, 21834-21845
23. □ Bridges, D., and Moorhead, G.B.G. (2004) *Science STKE* 242:re10
24. □ Woodcock, J.M., Murphy, J., Stomski, F.C., Berndt, M.C., and Lopez, A.F. (2003) *J. Biol. Chem.* **278**, 36323-36327
25. □ Xing, H., Zhang, S., Weinheimer, C., Kovacs, A., and Muslin, A.J. (2000) *EMBO J.* **19**, 349-358
26. □ Hamaguchi A, Suzuki, E., Murayama, K., Fujimura, T., Hikita, T., Iwabuchi, K., Handa, K., Withers, D.A., Masters, S.C., Fu, H., and Hakomori, S. (2003) *J. Biol. Chem.* **278**, 41557-41565
27. Brodie, S., and Blumberg, P.M. (2003) *Apoptosis* **8**, 19-27
28. Ma, Y., Pitson, S., Hercus, T., Murphy, J., and Lopez, A.F. (2005) *J. Biol. Chem.* **280**, 26011-26017