

## REVIEW ARTICLE

## Signal transduction of stress via ceramide

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The sphingomyelin (SM) pathway is a ubiquitous, evolutionarily conserved signalling system analogous to conventional systems such as the cAMP and phosphoinositide pathways. Ceramide, which serves as second messenger in this pathway, is generated from SM by the action of a neutral or acidic SMase, or by *de novo* synthesis co-ordinated through the enzyme ceramide synthase. A number of direct targets for ceramide action have now been identified, including ceramide-activated protein kinase, ceramide-activated protein phosphatase and protein kinase C $\zeta$ , which couple the SM pathway to well defined intracellular signalling cascades. The SM pathway induces differentiation, proliferation or growth arrest, depending on the cell type. Very often, however, the outcome of signalling through this pathway is apoptosis. Mammalian systems respond to diverse stresses

with ceramide generation, and recent studies show that yeast manifest a form of this response. Thus ceramide signalling is an older stress response system than the caspase/apoptotic death pathway, and hence these two pathways must have become linked later in evolution. Signalling of the stress response through ceramide appears to play a role in the development of human diseases, including ischaemia/reperfusion injury, insulin resistance and diabetes, atherogenesis, septic shock and ovarian failure. Further, ceramide signalling mediates the therapeutic effects of chemotherapy and radiation in some cells. An understanding of the mechanisms by which ceramide regulates physiological and pathological events in specific cells may provide new targets for pharmacological intervention.

## INTRODUCTION

The sphingomyelin (SM) pathway is a signalling system that is conserved from yeast to humans [1–4]. Ceramide, the central molecule in this pathway, serves as a second messenger for cellular functions ranging from proliferation and differentiation to growth arrest and apoptosis. The pleiotropic nature of ceramide signalling may be due to the fact that, in different cell types, it is linked to a variety of receptors. Further, ceramide engages different downstream effectors, depending on the cellular microenvironment, the concomitant activation of other second messengers and the activity of enzymes that convert ceramide into other metabolites. The magnitude of ceramide generation, the site and source of its generation, the phase of the cell cycle and the state of activation of transmodulating signals all appear to play a role in the final outcome. An improved understanding of the molecular basis of ceramide action may yield better insights into the pathogenesis of disease and provide novel strategies for therapeutic intervention in cancer as well as cardiovascular, neurodegenerative and autoimmune diseases. This review places in perspective the regulation of ceramide production, its role in signalling pathways and its effects on various organ systems.

## ENZYMES INVOLVED IN CERAMIDE GENERATION AND METABOLISM

Ceramide generation may involve hydrolysis of SM by various SMases or *de novo* synthesis of ceramide by a synthase [3,5–7].

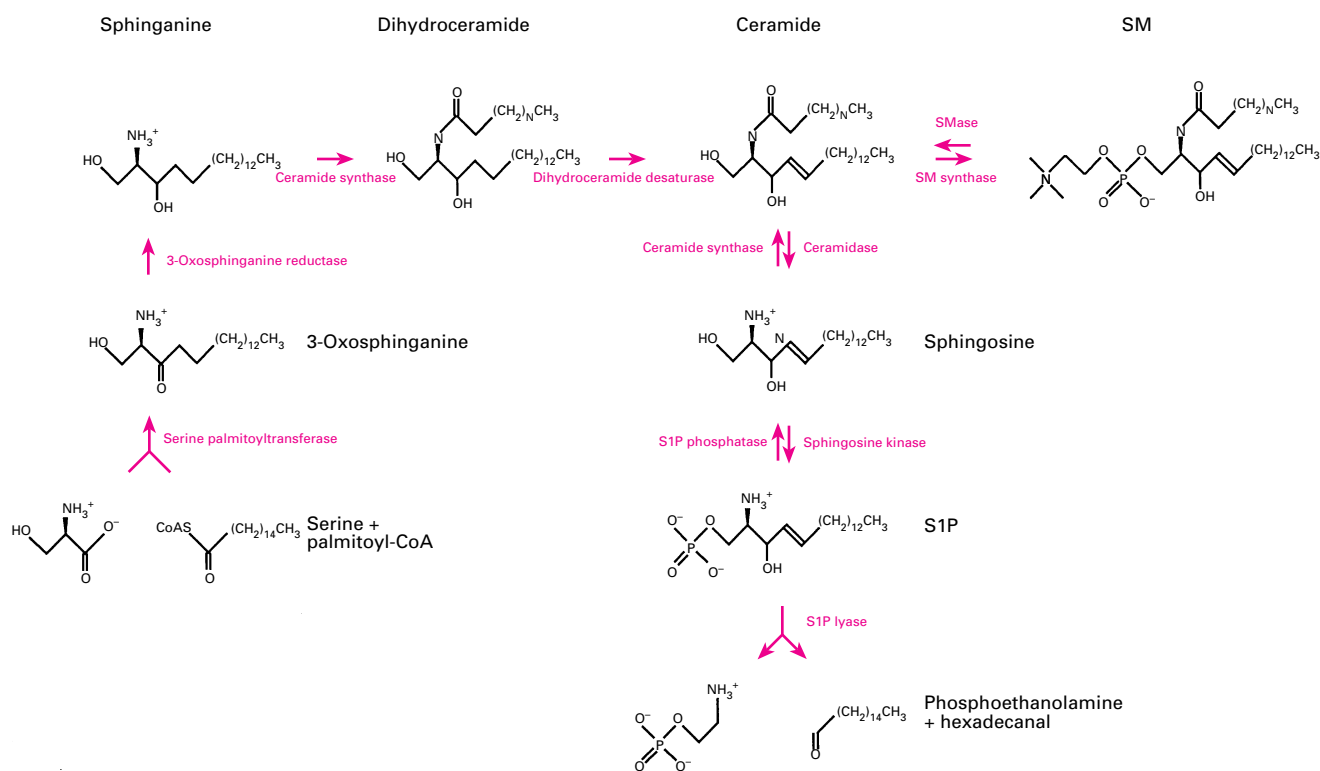
Some of these enzymes have been shown to be induced by physiological and environmental stimuli. Enzymes involved in ceramide generation are located in different subcellular compartments, and the site of function may play a role in the selective activation of effector complexes. Ceramide can also be generated via breakdown of glycosphingolipids by various hydrolases, although regulation of these enzymes by stress stimuli has not been reported.

The catabolic pathway for ceramide generation involves the action of SMases, i.e. SM-specific forms of phospholipase C, which hydrolyse the phosphodiester bond of SM yielding ceramide and phosphocholine (Figure 1) [3,5,6]. There are several isoforms of SMase, distinguished by different pH optima and hence referred to as acid, neutral or alkaline SMases. Both neutral and acid SMases are rapidly and transiently activated by diverse exogenous stimuli, leading to increases in ceramide levels in a time frame of seconds to minutes [1–4]. More prolonged activation of neutral SMase has also been reported [2].

Acid SMase was originally described as a lysosomal enzyme (pH optimum 4.5–5.0) which is defective in patients with Niemann–Pick (NP) disease [8,9]. Acid SMase activities have also been observed in the SM-rich plasma membrane microdomains (termed caveolae) of cells treated with interleukin-1 (IL)-1 [10] and nerve growth factor (NGF) [11]. Human and mouse acid SMases have been cloned and determined to be the product of a conserved gene [12,13]. The protein is generated as a 75 kDa precursor which is processed first to a 72 kDa form and then, by separate additional processing steps, to 70 and

Abbreviations used: ara-C, 1- $\beta$ -D-arabinofuranosylcytosine; BAEC, bovine aortic endothelial cells; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; C1P, ceramide 1-phosphate; CAPK, ceramide-activated protein kinase; CAPP, ceramide-activated protein phosphatase; CPT I, carnitine palmitoyltransferase I; DAG, diacylglycerol; DD, death domain; ERK, extracellular-signal-regulated kinase; FADD/MORT1, Fas-associated protein with a death domain; FB1, fumonisin B1; IFN, interferon; IL-1, interleukin-1; JNK, Jun kinase; KSR, Kinase Suppressor of Ras; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MPT, membrane permeability transition; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NGF, nerve growth factor; NP, Niemann–Pick; NT-3, neurotrophin-3; p75<sup>NTR</sup>, p75 receptor; PDGF, platelet-derived growth factor; PKC, protein kinase C; Rb, retinoblastoma; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; S1P, sphingosine 1-phosphate; SM, sphingomyelin; TAK1, transforming growth factor- $\beta$ -activated kinase; TNF, tumour necrosis factor; TRADD, TNF-receptor-associated protein with a death domain.

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**Figure 1** Spingolipid structures and enzymes of metabolism

57 kDa forms [14]. In transiently transfected COS-1 cells, Sandhoff and co-workers detected both the 75 kDa precursor and the 57 kDa protein in the culture medium [14,15]. Tabas and co-workers found that macrophages, fibroblasts and endothelial cells secrete substantial amounts of the high-molecular-mass form of acid SMase [16]. Further, the secreted form of acid SMase is  $Zn^{2+}$ -stimulable [17,18], whereas the lysosomal form is not. This may result from acquisition of cellular  $Zn^{2+}$  during the lysosomal targeting process [17]. Acid SMase secretion is increased by cytokine stimulation, perhaps by increasing the flux of the acid SMase precursor through the Golgi secretory pathway. Although the role of the secreted form of acid SMase has not been determined, it has been suggested that it may act in an autocrine or paracrine manner to hydrolyse cell surface SM and initiate signalling [17]. In this regard, an acidic environment enhances the activity of the secreted enzyme, but is not absolutely required [17].

The neutral SMases (pH optimum 7.4) have yet to be characterized at the molecular level. Acid SMase knock-out mice retain neutral SMase activity, indicating that the neutral forms are products of a distinct gene or genes [19,20]. One isoform appears to be membrane-bound and  $Mg^{2+}$ -dependent [21]. A cytosolic, cation-independent form has also been described [22]. Based on identity with neutral SMases from *Bacillus cereus* and *Leptospira interrogans*, Stoffel and co-workers recently published the sequence of a human enzyme, which when overexpressed in HEK293 cells resulted in a substantial increase in cellular neutral SMase activity [23]. This enzyme also displayed increased activity towards phosphatidylcholine. Whether this enzyme is actually a non-specific phosphodiesterase or a specific neutral SMase awaits direct characterization of the purified protein.

Alkaline SMase activity has been detected in the intestinal

mucosa and bile, and appears to be involved in digestion and mucosal cell proliferation [24,25]. This activity has not yet been characterized at the molecular level.

Ceramide can be synthesized *de novo* by condensation of serine and palmitoyl-CoA to form 3-oxosphinganine (Figure 1) [3,6]. This is reduced to dihydrosphingosine, acylated by ceramide synthase (sphinganine *N*-acyltransferase) to yield dihydroceramide and oxidized to ceramide by introduction of a *trans*-4,5 double bond. This pathway can be stimulated by drugs and ionizing radiation (see later) and requires several hours to generate detectable ceramide [26,27].

Once generated, ceramide may transiently accumulate or be converted into various metabolites. Phosphorylation by ceramide kinase [28,29] generates ceramide 1-phosphate (C1P), while deacylation by various ceramidases yields sphingosine, which may then be phosphorylated to sphingosine 1-phosphate (S1P) [3]. Ceramide may also be converted back into SM by transfer of phosphocholine from phosphatidylcholine to ceramide by the enzyme SM synthase [5]. Further, ceramide can be glycosylated by various enzymes in the Golgi apparatus to form complex glycosphingolipids. These various derivatives of ceramide may also serve as effector molecules.

Cellular ceramide content has generally been measured by one of four assays: the diacylglycerol (DAG) kinase assay [30], a method involving lipid charring [9], methods involving derivatization of ceramide followed by HPLC [31], and a variety of radiolabelling techniques [5]. Direct comparisons between these techniques have reproducibly yielded the same result [32]. Further, a corresponding and equimolar fall in SM levels can be measured simultaneously when the mechanism of ceramide generation involves SMases. The DAG kinase assay, which is the most commonly used method to measure ceramide, is based on

the ability of the *Escherichia coli* DAG kinase to utilize ceramide as a substrate [30]. When appropriately performed, all of the ceramide in a sample is converted into C1P. Hence the DAG kinase reaction is designed to progress to completion and therefore is not subject to activators contained within a biological sample [30]. In a recent report, Aebersold and colleagues suggested that ceramide measurements using the DAG kinase assay might not be accurate, and proposed a technique involving mass spectrometry [33]. These studies were most probably performed under conditions in which the DAG kinase assay did not proceed to completion, and hence was non-quantitative, yielding invalid conclusions. In our laboratory, a comparison of the DAG kinase assay with an HPLC method involving deacylation of ceramide and its derivatization with *o*-phthalaldehyde yielded a direct correlation between the two methods, with a coefficient of 0.94 [34]. Similarly, van Blitterswijk and colleagues have compared two methods for measuring ceramide, one involving metabolic labelling of ceramide with radiolabelled serine followed by TLC, and the other involving derivatization of ceramide with benzoate followed by HPLC [32]. These assays also yielded identical data.

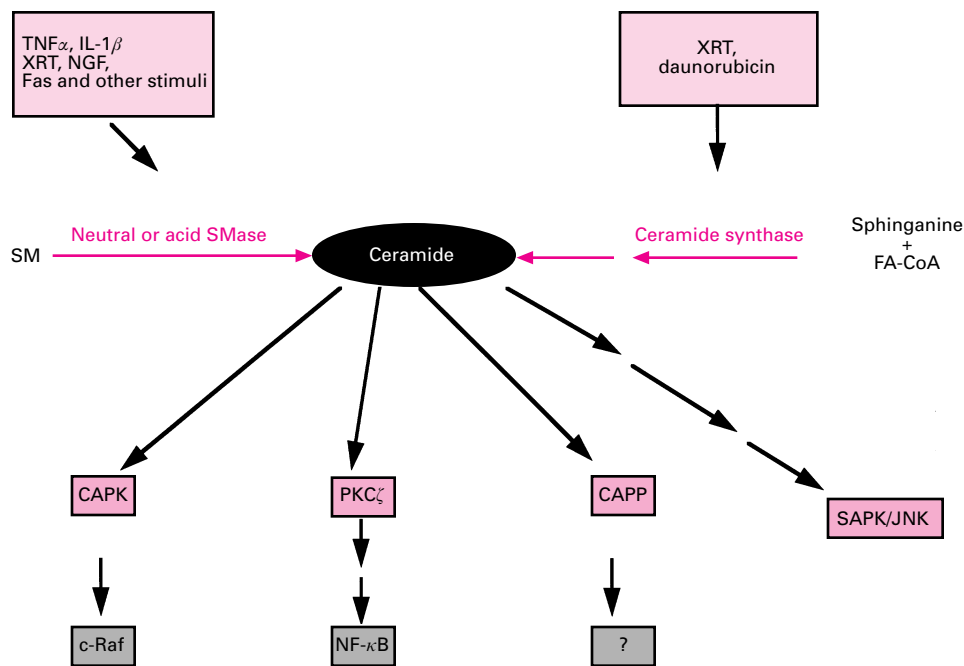
### MECHANISMS FOR SMase AND CERAMIDE SYNTHASE ACTIVATION

Numerous cellular stimuli signal via the SM pathway (Figure 2) [1–4,35]. Recent investigations have shown that agonists for receptors of the tumour necrosis factor (TNF) superfamily, and stress stimuli such as ionizing radiation, engage both neutral and acid SMases and activate apoptosis in some cell types, while in others they initiate anti-apoptotic signalling programmes [1–4,35].

The most comprehensive studies linking receptor activation to SMase activity and ceramide generation have utilized the p55

TNF $\alpha$  receptor as a model system. Kronke and co-workers reported that this receptor has a modular structure, with specific domains linked to different SMases [36]. By structure–function analysis of deletion mutants of the p55 TNF $\alpha$  receptor, these investigators were able to delineate an 11-amino-acid region of the receptor, adjacent to the death domain (DD), which is required for neutral SMase activation. This motif was termed the neutral SMase activation domain [37]. Further, using a library of overlapping peptides from the cytoplasmic portion of the TNF receptor to screen a cDNA expression library, a novel protein was isolated, which binds the neutral SMase activation domain and is required for neutral SMase activation [38]. This molecule, designated FAN (factor associated with neutral SMase activation), belongs to a family of regulatory proteins containing WD repeats, some of which are involved in signal transduction. Overexpression of FAN resulted in neutral SMase activation, whereas acid SMase activity was unaffected. The C-terminal cytosolic part of the 55 kDa TNF receptor, which contains the DD, appears to be required for acid SMase activation. The molecular mechanism for this interaction has not been determined and is probably indirect, perhaps via the DD adaptor proteins TRADD (TNF-receptor-associated DD protein) and FADD/MORT1 (Fas-associating protein with a DD) and a caspase-8-like protease (see below) [39–41]. Consistent with this notion, Schwandner et al. [42] recently reported that overexpression of TRADD and/or FADD enhanced the rapid activation (seconds to minutes) of acid SMase in response to TNF $\alpha$ , whereas caspase inhibitors blocked this event.

Treatment of cells with the chemotherapeutic drug daunorubicin resulted in ceramide generation over a period of hours in p388, HL-60 and U937 cells [26,43]. This increase in ceramide did not result from SMase activation, but rather from activation of the enzyme ceramide synthase [26,43] (It should be noted that,



**Figure 2** Mechanisms of ceramide generation and targets of ceramide action

Ceramide can be generated by catabolism of SM by either neutral or acid SMases, or by *de novo* synthesis. Synthesis involves the co-ordinate action of ceramide synthase, which catalyses the acylation of dihydrosphingosine to yield dihydroceramide, and dihydroceramide desaturase, which introduces the *trans*-4,5 double bond into the sphinganine backbone to yield ceramide [303]. Ceramide, once generated, stimulates ceramide-activated protein kinase to activate c-Raf-1, PKC $\zeta$  to signal through to NF- $\kappa$ B, and a ceramide-activated protein phosphatase (CAPP), the target of which is unknown. Ceramide also indirectly stimulates, in many systems, the SAPK/JNK cascade. Abbreviations: XRT, radiation; FA, fatty acid (see the Abbreviations footnote for other abbreviations).

**Table 1 Involvement of ceramide in signalling**

Abbreviations: ND, not determined; HUVEC, human umbilical vein endothelial cells; VCAM, vascular cell-adhesion molecule; ICAM, intracellular cell adhesion molecule; SEK, SAPK/ERK kinase; PARP, poly(ADP-ribose) polymerase; MBP, myelin basic protein; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; IRS-1, insulin receptor substrate-1; CMV, cytomegalovirus; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; GADD, growth arrest and DNA-damage-inducible; DA, dopaminergic; SMC, smooth muscle cells; PAF, platelet-activating factor; LIF, leukaemia inhibitory factor; CAPP, ceramide-activated protein phosphatase. For other abbreviations, refer to the Abbreviations footnote.

Stimulus	Cell	Proteins involved	Biological Effects	Refs.
TNF $\alpha$	HL-60	Neutral SMase, KSR/CAPK, Raf-1, MEK, MAPK	Monocytic differentiation	[95,97,98,290]
	COS-7	Raf-1, MEK, MAPK	ND	[98]
	HUVEC	Raf-1, MEK, MAPK	IL-6, IL-8, E-selectin, VCAM, ICAM induction	[122]
	A431	SMase, c-Jun	ND	[116]
	NIH 3T3, U937	SMase, PKC $\zeta$ , NF- $\kappa$ B	ND	[108]
	Astrocytes	JNK, HIV-1 nef, NF- $\kappa$ B	ND	[257,269]
	BAEC, U937, oligodendrocytes	SEK, JNK, c-Jun	Apoptosis	[49,228]
	Molt-4, oligodendrocytes	Caspase-3, PARP	Apoptosis	[135,229]
	Hepatocytes, cardiac cells	Mitochondrial complex III	Apoptosis	[119,155]
	Follicular granulosa cells	ND	Apoptosis	[199]
	U937, HL-60, MCF-7, HMEC-1 endothelial cells	Bcl-2	Inhibition of apoptosis	[40,147,289]
	MCF-7	Glutathione	Inhibition of apoptosis	[47]
	Hippocampal neurons	NF- $\kappa$ B	Protection from oxidative and excitotoxic shock	[160,241,243]
	Oligodendrocytes, astrocytes	ND	Inhibition of MBP/CNPase phosphorylation, differentiation	[265,266]
	L6 cells	SMase, MAPK	Inhibition of insulin-stimulated glucose uptake and glycogen synthesis	[126]
TNF + IFN $\gamma$	3T3-L1, 32D myeloid, Fao hepatoma	SMase, IRS-1	Insulin resistance	[191,192]
	Cardiac myocytes	SMase	Negative inotropy	[215]
	Oligodendrocytes, astrocytoma cells	ND	Inhibition of growth/differentiation, inhibition of CMV replication	[291]
TNF + IL-1	HEL 299	ND	Transcriptional down-regulation of M <sub>2</sub> muscarinic receptor	[292]
IL-1	EL-4 thymoma	Neutral SMase	IL-2 secretion	[67]
	Fibroblasts, granulosa cells	SMase, cyclo-oxygenase, prostaglandin endoperoxide synthase	Prostaglandin E <sub>2</sub> production, IL-6 production, inhibition of progesterone synthesis	[200,293]
	HUVEC	SMase	E-selectin up-regulation	[210]
	Pancreatic $\beta$ -cells	ND	Inhibition of insulin production	[196]
	FRTL-5 thyroid cells	SMase, PLA <sub>2</sub>	Inhibition of iodothyronine deiodinase	[294]
	Hepatocytes	SMase, ceramidase	$\alpha_1$ -Acid glycoprotein induction, cytochrome P <sub>450</sub> down-regulation	[295]
	Myocytes	ND	Ca <sup>2+</sup> channel current suppression	[296]
CD95	Human dendritic cells	ND	Differentiation, impairment of antigen uptake and presentation	[297]
	Jurkat	Ras, SMase, Rac-1, JNK, p38 kinase, GADD153, SMase, caspase-3	Apoptosis	[32,85,170]
	Jurkat, U937, HUT78, L1210	Acid SMase	Apoptosis	[142,171]
	Oligodendrocytes, microglia	Fas-L	Apoptosis	[231]
	SKW6.4B lymphoblastoid cells, MUTU-BL	SMase or PKC	Apoptosis or inhibition of apoptosis respectively	[172]
NGF	BJAB, MCF-7	FADD	Apoptosis	[79]
	T9 glioma	SMase	Anti-proliferation	[225]
	Oligodendrocytes	SMase, JNK	Apoptosis	[226,234]
	PC12	Sphingosine kinase	Neurofilament expression, neuroprotection	[298]
NGF, BDNF, NT-3, NT-5	Mesencephalic DA neurons	SMase	Dopamine release	[236]
	p75-NIH 3T3, PC12, DA neurons	SMase, TrkA/B/C	Modulation of Trk signalling	[221,236,237]
	BAEC, B-lymphoblasts	Acid SMase, SEK, JNK	Apoptosis	[49,81,84]
	WEHI-231, SQ-20B	Neutral SMase, PKC	Apoptosis	[51,276,299]
$\gamma$ -Radiation	U937, HL-60	Bcl-XL, Bcl-2, CD40	Inhibition of apoptosis	[289,300,301]
	BAEC	SMase, SEK, JNK	Apoptosis	[49]
	Granulosa cells	ND	Apoptosis	[199]
Stress (UV, heat shock, H <sub>2</sub> O <sub>2</sub> )	Hippocampal neurons	NF- $\kappa$ B	Growth/survival	[160,241]
	P388, U937	Ceramide synthase	Apoptosis	[26]
Anthracyclines	HL-60, U937	SMase	Apoptosis	[44]
	ALL-697	ND	Apoptosis	[53]
Vincristine	ALL-697	ND	Apoptosis	[53]
Ara-C	Jurkat, HL-60	ND	Apoptosis	[54,55]
Camptothecin, CPT11	4B1 fibroblasts	Ceramide synthase, caspase-3	Apoptosis	[56]

**Table 1** (Cont.)

Stimulus	Cell	Proteins involved	Biological Effects	Refs.
PDGF	NIH 3T3, vascular SMC, glomerular cells	SMase, ceramidase	Proliferation	[62]
CD28	Jurkat	SMase, MAPK	Proliferation	[123,124]
Anti-IgM	WEHI-231	ND	Apoptosis	[51,175]
LPS + PAF	Macrophages	Ceramide synthase, group V PLA <sub>2</sub>	Inflammation	[180]
Oxidized LDL	Vascular SMC	Zn <sup>2+</sup> -activated acid SMase	SMC proliferation, LDL aggregation, foam cell formation	[204,205,213,214]
Phenylephrine	Aorta	SMase	Relaxation	[215]
Serum withdrawal	Molt-4	Rb	G0/G1 growth arrest	[57,58]
ND	Raji lymphoma	Rb	G0/G1 growth arrest	[302]
ND	Neutrophils	MAPK	Inhibition of respiratory burst and phagocytosis	[54,80]
ND	HUVEC	SMase	Induction of plasminogen activator inhibitor-1	[211]
ND	Astrocytes	PKC $\xi$	NGF release	[260,263]
		SMase, MEK, NF- $\kappa$ B	Nitric oxide induction	[261]
ND	Schwann cells	NF- $\kappa$ B	LIF transcription	[260]
ND	Hippocampal neurons	ND	Bipotent; growth and apoptosis	[244,252]
ND	Various neurons	ND	Apoptosis	[241–245]
ND	Sympathetic neurons	SMase	Protects from NGF-withdrawal-induced death	[240]
ND	PC12	Calpain I	Inhibits NGF-induced differentiation	[245,246]
ND	Yeast	CAPP	Growth arrest	[72,113]

in some cells, daunorubicin may stimulate early SMase activation, independent of the prolonged effects on ceramide synthase [44].) Further, the fungal toxin fumonisin B1 (FB1), which Merrill and co-workers have documented as a specific inhibitor of ceramide synthase activity [45], blocked daunorubicin-induced synthase activation, ceramide generation and cell death. The mechanisms of activation of the enzyme by daunorubicin has not yet been determined, although a signal from damaged DNA may be involved. Consistent with this hypothesis, recent studies show that metabolic incorporation of <sup>125</sup>I-labelled 5-iodo-2-deoxyuridine, which produces DNA double-strand breaks, signalled *de novo* ceramide synthesis by post-translational activation of ceramide synthase [46].

Recently, Hannun and co-workers showed that neutral SMase is inhibited by physiological concentrations of glutathione [47,48]. Further, these investigators provided evidence that a fall in the glutathione level, which often occurs in response to oxidative stress stimuli, might trigger prolonged neutral SMase activation, resulting in sustained ceramide generation and feed-forward signalling of apoptosis [47,48].

### EFFECTS OF CERAMIDE ON CELL FATE

A variety of physiological signals, such as those generated by cytokines and growth factors, induce changes in ceramide levels [35]. Ceramide is also generated in response to stress stimuli, such as ionizing and UV radiation [49–52], chemotherapeutic drugs [26,43,44,53–56], serum withdrawal [57,58] or oxidative stress [49]. Recent data show that this stress response system is evolutionarily conserved in yeast [59,60]. Once generated, the ceramide signal affects multiple aspects of cellular function, including proliferation, differentiation, growth arrest and death.

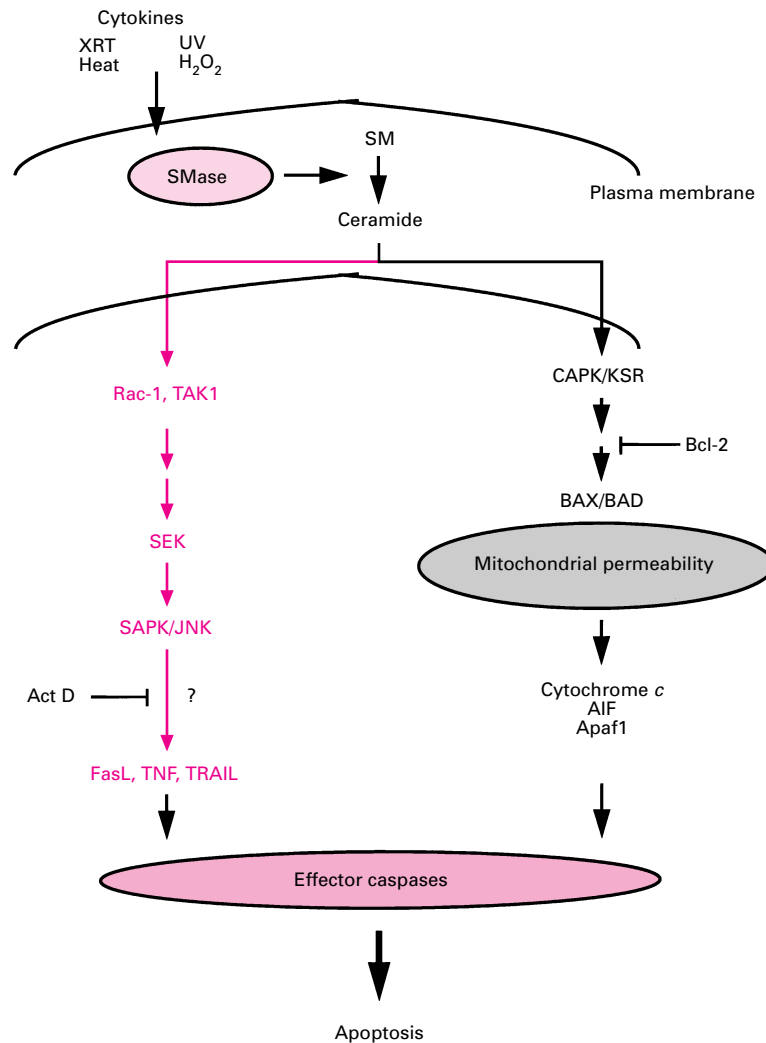
#### Proliferation

S1P is synthesized rapidly in response to mitogenic signals such as platelet-derived growth factor (PDGF) or serum in many mammalian cells [3]. Further, analogues of S1P mimic this proliferative response, and antagonize ceramide-induced apo-

ptosis [3]. This may result, in part, from transmodulation of pro-apoptotic ceramide signalling through the Jun kinase (JNK) cascade [49,61] (see later) to the mitogen-activated protein kinase (MAPK) cascade, which is often anti-apoptotic [61]. Based on this information, Spiegel and co-workers [89] proposed the existence of a ceramide/sphingosine rheostat as a mechanism for co-ordinately regulating proliferation and apoptosis through sphingolipids. In this model, a balance exists between ceramide and S1P that is regulated by ceramidase, the enzyme that converts ceramide into free sphingosine (Figure 1). Sphingosine, once generated, can be rapidly phosphorylated to S1P by sphingosine kinase. Consistent with this model, during PDGF-induced proliferation of fibroblasts, vascular smooth muscle cells and glomerular mesangial cells, SMase activation and ceramide generation were followed by ceramidase activation, and inhibition of ceramidase with *N*-oleoylethanolamine blocked PDGF-induced proliferation, in part [62].

A variant of this mechanism exists in yeast, where increased sphingolipid synthesis appears to be required for the development of thermotolerance, i.e. the capacity of yeast to adapt to heat stress and resume growth. In yeast, heat shock is rapidly followed by sphingoid base synthesis and subsequently ceramide generation. Mutants incapable of synthesizing these sphingolipids fail to grow at elevated temperature, and sphingoid base analogues bypass the defect, restoring growth. Further, mutants defective in sphingoid base 1-phosphate phosphatase [63] or dihydrosphingosine 1-phosphate lyase [64], enzymes required for degradation of phosphorylated sphingoid bases, contain elevated dihydrosphingosine 1-phosphate levels, and display resistance to heat stress. Although definitive studies are not yet available, preliminary information suggests, as in mammalian systems, that ceramide signals anti-proliferative or lethal responses during the yeast heat shock response (see below). It should be noted that a stimulated elevation of phosphorylated sphingoid bases in response to heat has not yet been reported in yeast.

Other metabolites of ceramide may also signal proliferative responses in some cells. C1P, a product of ceramide phosphorylation by ceramide kinase, also stimulated proliferation of NIH 3T3 fibroblasts [65]. In this regard, treatment with natural



**Figure 3** Ceramide signals apoptosis by transcription-dependent and -independent mechanisms

Ceramide-induced apoptosis may occur via two independent mechanisms. Ceramide signals, perhaps through the JNK cascade, the transcriptional regulation of gene products, such as Fas ligand (FasL) or TNF $\alpha$ , that mediate the death response [50,85; K.-M. Debatin, personal communication). Alternatively, ceramide induces apoptosis directly through a mechanism inhibitable by anti-apoptotic Bcl-2 family members [135,144,153]. This transcription-independent pathway for induction of apoptosis has been observed in cytoplasts [151] and was recently reconstituted in a cell-free system [153]. The target for ceramide and the signalling system involved in this latter death paradigm are unknown. Abbreviations: XRT, radiation; Act D, actinomycin D; TRAIL, TNF-related apoptosis-inducing ligand; AIF, apoptosis-inducing factor (see the Abbreviations footnote for other abbreviations).

CIP induced DNA synthesis and expression of proliferating-cell nuclear antigen, a non-histone nuclear protein involved in cell cycle progression.

### Differentiated functions

Stimulation of cells with TNF $\alpha$ , IL-1 $\beta$  or interferon  $\gamma$  (IFN $\gamma$ ) promotes ceramide generation and differentiation in numerous cell types [66–69]. For instance, TNF $\alpha$ -treated HL-60 promyelocytic cells undergo monocytic differentiation, and ceramide analogues mimic this response [70]. Similarly, IL-1 $\beta$ -induced differentiation of EL-4 thymoma cells was accompanied by ceramide generation, and exogenous ceramide mimicked effects of IL-1 $\beta$ , such as co-stimulation of IL-2 secretion [67]. A survey of cells responding to ceramide with differentiated function is included in Table 1.

### Growth arrest

Ceramide has been shown to play a role in growth arrest subsequent to serum withdrawal [57,58]. In cultured Molt-4 leukaemia cells, serum withdrawal leads to a 10–15-fold elevation in ceramide levels, accompanied by arrest at the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle in 80% of the cells. This effect was correlated with dephosphorylation of the retinoblastoma (Rb) gene product, generating the form of the protein implicated in inhibition of cell cycle progression [57,58]. Addition of C<sub>6</sub>-ceramide, a synthetic ceramide analogue, replicated the G<sub>0</sub>/G<sub>1</sub> arrest and Rb dephosphorylation effects of serum withdrawal. Additionally, cells either partially deficient in Rb or expressing proteins that sequester the Rb protein were resistant to ceramide-induced growth arrest. An anti-proliferative response to ceramide has also been shown to result in G<sub>1</sub> arrest in yeast [71], perhaps

mediated via a ceramide-activated protein phosphatase (CAPP) [72] (see below). In addition, ceramide, which accumulates in mutants lacking phosphatidylinositol:ceramide phosphoinositoltransferase or after inhibition of this enzyme by the anti-fungal agent aureobasidin A, mediates a form of non-apoptotic cell death in *Saccharomyces cerevisiae* [73].

### Apoptosis

In general, apoptosis can be divided into three phases: an initiation, a commitment and an effector phase [74]. Ceramide is generated and acts as a second messenger during the initiation phase. Apoptosis occurring in response to stimulation of the TNF superfamily receptors, such as the p55 TNF $\alpha$  receptor and CD95/Apo-1/Fas, has an initiation phase that involves assembly of a signalling complex of adaptor proteins via the DDs of these receptors [75–78]. Ceramide appears to be generated subsequent to formation of this complex in some cell systems [41,42,79]. In apoptosis induced by stresses such as ionizing and UV radiation, exposure to oxygen radicals and heat shock, the events occurring during the initiation phase have not been completely determined, but appear to involve the generation of ceramide by a mechanism independent of the DD signalling complex [80]. While apoptosis can occur independent of ceramide generation, in certain tissues and for certain stimuli ceramide generation appears to be required for optimal apoptosis. For instance, acid SMase knock-out mice, which are unable to elevate ceramide levels in response to ionizing radiation or lipopolysaccharide (LPS)/TNF $\alpha$ , are resistant to endothelial cell apoptosis in response to these stresses [81]. Similarly, Debatin and co-workers found that fibroblasts from patients with NP disease were deficient in UV- and anthracycline-induced ceramide generation and apoptosis [50]. Giacchia and co-workers [81a] also found that NP cells were defective in ionizing-radiation-induced ceramide generation and death. For CD95-induced death, however, NP cells have produced conflicting data. While Testi and co-workers claim that acid SMase is required [82], Boesen-de Cock et al. suggest that neutral SMase is involved [83]. It is well documented that agents such as phorbol esters, basic fibroblast growth factor (bFGF) or serum affect the set-point of this system, regulating the intracellular ceramide level and the responsiveness to stress, and perhaps accounting for differences between groups with regard to ceramide signalling [2]. Ceramide may regulate apoptosis by two different mechanisms, one involving transcriptional activation of the JNK pathway [49,84–92] and the other via alteration of mitochondrial function (Figure 3) (see below). The second and third phases, common to most forms of apoptosis, consist of a commitment phase characterized by alteration of mitochondrial function and recruitment of a cascade of effector caspases, and an effector phase during which key cellular proteins are inactivated via cleavage, and organelles are degraded and packaged.

### DIRECT TARGETS OF CERAMIDE ACTION

The exact mechanisms mediating the pleiotropic activities of ceramide are for the most part unknown, although they appear to depend on direct targets for ceramide action. The known targets for ceramide are ceramide-activated protein kinase (CAPK), protein kinase C $\zeta$  (PKC $\zeta$ ) and CAPP (Figure 2). In addition, ceramide interacts with several signalling systems, including the MAPK, JNK, caspase and mitochondrial signalling systems.

### CAPK

CAPK was originally defined as a 97 kDa membrane-associated, proline-directed protein kinase with a substrate recognition motif of Xaa-Thr-Leu-Pro-Xaa [93–95]. CAPK is stimulated by TNF $\alpha$  treatment of HL-60 cells [96] and activates the MAPK pathway via phosphorylation of c-Raf-1 [97]. Zhang et al. [98] compared CAPK biochemically with KSR, the recently cloned kinase suppressor of Ras, and concluded that they are identical. KSR was originally defined in genetic screens in *Drosophila* and *Caenorhabditis elegans* as downstream of Ras, and upstream of or parallel to Raf-1 [99–101]. Based on similarity of size, the molecular ordering of KSR upstream of Raf-1 and the presence of a putative lipid binding cassette in KSR, it was hypothesized that CAPK was KSR. Consistent with this supposition, over-expression of mouse KSR cDNA in COS-7 cells led to constitutive activation of c-Raf-1 [98]. Further, treatment of transfected cells with TNF $\alpha$  or synthetic ceramide analogue enhanced KSR autophosphorylation, and increased its ability to phosphorylate and activate Raf-1. Like CAPK, natural ceramide, but not other lipids, stimulated KSR to autophosphorylate, and transactivate Raf-1 *in vitro* [98]. KSR phosphorylated Raf-1 on the same site, Thr<sup>269</sup>, as CAPK. In contrast, kinase-inactive KSR did not activate c-Raf-1, and mutation of the CAPK phosphorylation sites on c-Raf-1 blocked KSR/CAPK signalling.

Other groups have arrived at different conclusions as to the mechanism by which KSR acts. Morrison and co-workers [102] and Muslin and co-workers [103], like Zhang et al. [98], found that KSR binds to and activates Raf-1, enhancing signalling through the MAPK cascade. This interaction was reported as being required for *Xenopus laevis* oocyte maturation, cellular transformation and *Drosophila* eye development, although there is disagreement as to whether the kinase domain of KSR is obligatory [104]. In contrast, Williams and co-workers [105] and Eychene and co-workers [106] report that KSR binds to and functionally inactivates MEK1 (MAPK/ERK kinase 1, where ERK is extracellular-signal-regulated kinase), blocking signalling through MAPK and attenuating Ras-induced transformation and serum-induced mitogenesis. Perhaps the discrepancies in these data reflect the induction of apoptosis. Karim and Rubin [107] have shown recently that overexpression of v12-ras at low gene doses induces hyperproliferation of cells in *Drosophila* wing and eye discs, whereas higher levels of expression induce apoptosis. Both events require functional KSR, Raf, MEK and MAPK, as loss-of-function mutants suppress lethality and disc overgrowth. In some of the systems described above, inadvertent apoptosis might select for a population in which alternative actions of KSR predominate. Consistent with this observation, recent studies have shown that overexpression of KSR induces apoptosis in COS-7 cells that express the pro-apoptotic Bcl-2 family member BAD [154].

### PKC $\zeta$

PKC $\zeta$  is an atypical PKC isoform which is insensitive to DAG and phorbol esters, but responsive to ceramide. Treatment of U937 cells or NIH 3T3 fibroblasts with TNF $\alpha$ , SMase or ceramide analogue increased PKC $\zeta$  phosphorylation and activity. Radiolabelled ceramide bound to and activated PKC $\zeta$  in a biphasic manner, with concentrations of ceramide as low as 0.5 nM leading to a 4-fold increase in autophosphorylation of PKC $\zeta$ , whereas concentrations above 60 nM caused down-regulation [108]. PKC $\zeta$  may play a role in TNF-induced activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) via SMase in some cells, since dominant-negative kinase-defective PKC $\zeta$  blocked SMase-induced NF- $\kappa$ B activation in NIH 3T3 fibroblasts [109]. This

event may be cell-type-specific, as SMase does not appear to be involved in TNF-induced NF- $\kappa$ B activation in mouse embryo fibroblasts [110], where the more well documented TRAF-2-dependent pathway for NF- $\kappa$ B activation is most probably operative (where TRAF is TNF-receptor-associated factor). The recent demonstration that PKC $\zeta$  moves to the nucleus in response to ceramide elevation suggests that it may regulate the transcriptional apparatus during ceramide signalling [111].

### CAPP

CAPP belongs to the protein phosphatase 2A family of serine/threonine phosphatases [112–114]. These phosphatases exist as heterotrimers in which subunits A and B are regulatory and subunit C is catalytic. The B subunit is required for activation by ceramide. A yeast homologue of CAPP has been described by Nickels and Broach [72], consisting of regulatory subunits encoded by the genes *TPD3* and *CDC55* and a catalytic subunit encoded by *SIT4*. These gene products mediate an anti-proliferative response to ceramide, arresting cells at the G1 phase of the cell cycle. It has also been proposed that CAPP plays a role in down-regulation of *c-myc* expression in mammalian cells [115]. Further, in TNF $\alpha$ -treated A431 cells, rapid SM hydrolysis was accompanied by c-Jun dephosphorylation [116]. This effect was mimicked by exogenous SMase or synthetic ceramide analogues, and a partially purified CAPP preparation dephosphorylated c-Jun *in vitro*. Since JNK phosphorylates c-Jun while promoting apoptosis in some cells [49,61], these data suggest that CAPP may act as an antagonistic signal, inhibiting ceramide-mediated apoptosis by reversing effects on JNK.

### Other targets

Ceramide has been reported to directly activate c-Raf-1 [117,118] and inhibit purified mitochondrial complex III [119]. The relevance of these interactions to ceramide signalling is at present uncertain.

## INTERACTION OF CERAMIDE WITH SIGNALLING CASCADES

### Ceramide and MAPK

The MAPK pathway, also known as the ERK pathway, usually mediates growth and inflammatory signals [120]. Ceramide was shown to regulate the MAPK pathway in a variety of cell types. TNF $\alpha$ -induced monocytic differentiation of HL-60 cells involves activation of the p42 MAPK [121]. Further, in COS-7 cells, synthetic ceramide analogues and exogenous SMase mimicked the effect of TNF $\alpha$  via successive activation events involving KSR/CAPK, c-Raf-1, MEK and MAPK [97]. Ceramide also mimicked TNF $\alpha$ -induced activation of c-Raf-1, MEK and MAPK in human umbilical vein endothelial cells, leading to a pro-inflammatory response [122]. In T cells, stimulation of CD28 engages the SMase signalling system for activation of the MAPK cascade which is involved in proliferation [123,124]. In contrast ceramide-mediated inhibition of MAPKs has been implicated in antagonism of the respiratory burst and antibody-dependent phagocytosis in neutrophils [125], as well as in TNF $\alpha$ -mediated inhibition of insulin-stimulated glucose uptake and glycogen synthesis [126]. Thus ceramide may either activate or inhibit the MAPK pathway, depending on the cell type.

### Ceramide and JNKs

While ceramide signals inflammation via the MAPK pathway, it appears to trigger apoptosis via the JNK [also called stress-activated protein kinase (SAPK)] pathway. The JNK pathway

is activated in response to diverse stresses, including cytokines, UV and ionizing radiation, serum deprivation, osmotic shock and ischaemic injury [120], and leads to phosphorylation of transcription factors such as c-Jun, activating transcription factor-2 (ATF-2) and CHOP/GADD153. The JNK family of proteins is encoded by three distinct genes, resulting in 10 alternatively spliced isoforms which differ in their effector functions [127]. For instance, JNK1 mediated UV-induced apoptosis in small-cell lung cancer cells, whereas JNK2 was without effect [128].

The role of ceramide in activating the JNK pathway has been demonstrated in several studies. Verheij et al. [49] showed that exposure of bovine aortic endothelial cells (BAEC) and U937 lymphoblastic cells to TNF $\alpha$  or environmental stresses resulted in rapid ceramide generation, activation of the JNK pathway and apoptosis. Ceramide analogues similarly stimulated JNK activation, while disruption of the pathway with dominant-negative mutants of SEK1 (SAPK/ERK kinase 1)/MKK4 (MAPK kinase 4) or c-Jun blocked TNF $\alpha$ -, stress- and ceramide-induced apoptosis. Dong and co-workers [84] defined an obligatory role for ceramide in UV-induced JNK activation, since B cells from NP patients failed to undergo JNK activation yet retained responsiveness to ceramide analogues. Ceramide generation may lead to JNK activation by two potential mechanisms, either via transforming-growth-factor  $\beta$ -activated kinase (TAK1) or via the small G-protein Rac-1. Shirakabe et al. reported that TAK1 is activated in response to ceramide or stresses that lead to ceramide generation, and that a kinase-negative TAK1 blocked ceramide-induced JNK activation [87]. Gulbins and co-workers showed that, in Jurkat cells, CD95 activation or ceramide treatment resulted in sequential activation of Ras, Rac-1 and the JNK pathway [85]. Expression of dominant-negative N17Ras or N17Rac-1 completely inhibited ceramide- and CD95-induced JNK activation and apoptosis, but not CD95-induced ceramide generation, suggesting that Ras and Rac-1 are required for ceramide-mediated JNK activation and apoptosis. Thus, in response to stress stimuli, ceramide may utilize a variety of mediators to activate the JNK pathway. It should be noted that there are multiple pathways to JNK activation, most of which do not involve ceramide and may play no role in apoptosis or may even provide anti-apoptotic protection to cells [129]. Until better reagents are available to distinguish between JNK isoforms, and the regulation of the proximal signals becomes apparent, it will not be possible to definitively assign the role of this system in the induction of apoptosis.

### Ceramide and caspases

Apoptosis in mammalian systems occurs by recruitment of a cascade of caspase proteases. Caspases are divided functionally into initiator caspases, such as caspase-2, -8, -9 and -10, which couple to cytokine receptors of the TNF superfamily and link to effector caspases such as caspase-3 and -7, which commit cells to the death programme [39]. Initiator caspases appear to play no role in programmed cell death in response to environmental stress [80], except in instances where environmental stresses signal up-regulation of TNF receptor superfamily members or their ligands [50]. There is currently abundant evidence in mammalian systems that ceramide functions proximal to effector caspases [32,40,130–135]. Activation of effector caspases by ceramide is inhibited by Bcl-2 overexpression in numerous systems (see below). The use of caspase inhibitors provides a somewhat more confusing picture. In general, ceramide-induced death is inhibited by agents that block caspase-3-like proteases, such as the viral inhibitor p35 or the peptide caspase inhibitor Asp-Glu-Val-Asp-aldehyde, and in some cases by benzyloxy-



carbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone [32,130–133, 136–138], whereas inhibitors of caspase-1- or caspase-8-like proteases, such as Tyr-Val-Ala-Asp or crmA, fail to block ceramide-induced death [40,130,133,136,139]. Some exceptions to this observation do exist, however [140,141].

Recent studies support a role for initiator caspases in activating SMase during signalling of apoptosis in response to activation of the TNF receptor and CD95. Deletions of the DD region of the 55 kDa TNF receptor [36] and CD95 [142], as well as over-expression of dominant-negative FADD/MORT1 [79], blocked ligand-induced ceramide generation and apoptosis. Similarly, crmA inhibited both ceramide generation and cell death in response to TNF $\alpha$ , but did not affect death induced by ceramide analogues [40]. Overexpression of TRADD and FADD enhanced TNF $\alpha$ -induced activation of acid SMase, but not neutral SMase, while TRAF2 and receptor-interacting protein (RIP) were without effect [42]. Further, in at least one study, CD95-induced apoptosis was attenuated in acid-SMase-deficient NP lymphoblasts, a defect reversed upon mannose-receptor-mediated transfer of acid SMase into the cells [82]. All these lines of evidence suggest that ceramide generation occurs subsequent to activation of a crmA-inhibitable initiator protease, perhaps caspase-8 or -10, and prior to effector caspase activation.

### Ceramide and mitochondria

Recent studies have defined a prominent role for mitochondria in the commitment phase of the apoptotic response. Mitochondria may function to bind Apaf1, release cytochrome *c*, generate reactive oxygen species (ROS) and/or undergo permeability transition to mediate apoptosis. Signalling of the apoptotic response through mitochondria is regulated in many instances by Bcl-2 and family members. In every report so far, Bcl-2 inhibits ceramide-induced apoptosis [40,53,135,136,139,143–149].

Two distinct mitochondrial events appear to be related to ceramide action: permeability transition and ROS generation. Induction of apoptosis is frequently accompanied by membrane permeability transition (MPT), which is associated with the opening of large pores in the mitochondrial inner membrane, allowing free diffusion of substances with a molecular mass of less than 1.5 kDa [150]. MPT results in loss of mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) and, according to Kroemer and co-workers, precedes phosphatidylserine externalization in the outer leaflet of the plasma membrane, generation of ROS and DNA fragmentation [149,151]. MPT results in release of proteases and other factors from mitochondria that amplify apoptosis. Induction of MPT in intact cells by agents such as TNF $\alpha$  or ionizing radiation is mimicked in isolated mitochondria by a cytoplasmic fraction from ceramide-treated cells [151–153]. Since MPT can be signalled in cytoplasts, ceramide-induced MPT would appear to be a transcriptionally independent event [146,151]. Preliminary studies suggest that one form of this event is mediated by BAD and signalled through KSR/CAPK [154]. Kroemer and co-workers distinguished ceramide signalling of MPT from upstream caspase signalling of this event, as only the ceramide effect is inhibited by Bcl-2 [153].

Ceramide also has direct effects on isolated mitochondria, resulting in generation of ROS, perhaps independent of MPT [119,155]. The site of ceramide action was shown to be at respiratory complex III, since inhibition of electron transport through complexes I and II blocked ceramide action, whereas inhibition of complex III activity potentiated the ceramide effect. Consistent with this hypothesis, Guduz et al. showed that ceramide inactivated purified complex III *in vitro* [119]. Further, TNF $\alpha$  treatment was shown in one study to increase the level of

ceramide in the mitochondria [155], suggesting that this event may be of physiological importance.

Ceramide may play additional roles at the mitochondria during apoptosis. In a screen for genes involved in apoptosis, Paumen et al. isolated carnitine palmitoyltransferase I (CPT I), an enzyme located in the mitochondrial outer membrane, which catalyses the transfer of long-chain fatty acids into the mitochondria for  $\beta$ -oxidation [156]. In the presence of a CPT I inhibitor, treatment of cells with fatty acids such as palmitate, which serve as precursors of *de novo* ceramide synthesis, led to ceramide generation and apoptosis. The ceramide synthase inhibitor FBI blocked these events. CPT I has also been shown to bind Bcl-2, suggesting that these two proteins may regulate each other's activity [157].

Recently, Obeid and co-workers reported that ceramide analogues induced cytochrome *c* release from mitochondria and that this event was inhibited by Bcl-2 [158]. Whether cytochrome *c* release results from MPT, effects on the electron transport system or stimulated secretion [159] is at present unknown.

## BIOLOGICAL EFFECTS OF CERAMIDE ON VARIOUS ORGAN SYSTEMS

### Immune system

Ceramide signalling contributes to several aspects of immune function [4]. Ceramide serves second messenger function for the cytokines TNF $\alpha$  [3,5–7,66,160,161], IL-1 $\beta$  [10,67,68,81, 162–168] and IFN $\gamma$  [69,163,169], and has been implicated in signalling by several lymphocyte surface proteins. Ligation of CD28, which functions as a co-stimulatory signal for T cell proliferation, utilizes the SMase signalling system for activation of the MAPK cascade [123,124]. As discussed above, stimulation of CD95, which signals deletion of autoreactive T cells and mediates cytolytic T-cell-induced death, leads to rapid and/or prolonged ceramide generation in numerous cell types [32,79, 85,141,142,165,167,170–174]. In the B-lymphocyte cell line WEHI 231, treatment with anti-IgM antibody induced apoptosis via ceramide generation, while an apoptosis-resistant subline was found to be deficient in this response [175].

Ceramide signalling also contributes to a variety of inflammatory responses. Stimulation of adherent neutrophils with formyl-Met-Leu-Phe resulted in ceramide generation, and exogenous ceramide inhibited both the respiratory burst and antibody-dependent phagocytosis [125,176]. The latter correlated with an inhibition of MAPK phosphorylation and activation. Ceramide also inhibited phorbol ester- and TNF $\alpha$ -induced superoxide release from neutrophils [177,178]. Thus ceramide tends to attenuate the inflammatory response of neutrophils. Alternatively, ceramide signals the generation of the pro-inflammatory lipid arachidonic acid in some cell types. In L929 fibrosarcoma cells, ceramide signalled an increase in the levels of cytosolic phospholipase A<sub>2</sub> and cyclo-oxygenase-2 mRNAs [179]. Moreover, treatment of macrophages with a combination of bacterial LPS and platelet-activating factor induced ceramide generation via ceramide synthase, and triggered arachidonate signalling via group V secretory phospholipase A<sub>2</sub> [180].

Acid SMase may also be required for infection of mucosal cells by *Neisseria gonorrhoeae* [181]. Invasion of fibroblasts and epithelial cells by *N. gonorrhoeae* was accompanied by SM hydrolysis to ceramide, due to activation of acid SMase. Infection was prevented by imipramine-induced degradation or antisense inactivation of acid SMase. Further, fibroblasts from NP patients were resistant to infection, while transfection of acid SMase restored the capacity for *N. gonorrhoeae* uptake. These investigations demonstrate a requirement for ceramide, generated by

acid SMase, in infection by *N. gonorrhoeae* and perhaps other bacterial pathogens.

In HIV-infected individuals, lymphocyte apoptosis may signal T cell depletion and disease progression. Cifone and colleagues have reported that circulating populations of CD4+ and CD8+ T cells from HIV-infected patients display large increases in ceramide content and apoptosis as compared with a normal population [182,183]. Similarly, ceramide levels are markedly increased in HIV-infected CEM cells [184]. Treatment of HIV-infected HL-60 cells [185] or the latently infected myelomonocytic cell lines U-111B and OM-10.1 [186] with ceramide analogues profoundly increased viral reproduction. Alternatively, treatment of HIV-infected patients with L-carnitine, which reduced acid SMase activity [182], significantly reduced ceramide levels in peripheral blood mononuclear cells, and correlated with a decrease in the number of apoptotic CD4+ T-cells [183]. Thus ceramide mediates immune and inflammatory activities, by signalling proliferation, differentiated functions, apoptosis or viral replication, depending on the cell types involved.

### Endocrine system

Ceramide mediates the effects of cytokines on endocrine function. TNF $\alpha$  produced by adipocytes has been implicated in insulin resistance associated with obesity and non-insulin-dependent diabetes mellitus [187–189]. Numerous reports document that ceramide mimics this event, through multiple, perhaps coordinate, mechanisms [126,190–194]. TNF $\alpha$ , ceramide analogues and exogenous SMase stimulate serine phosphorylation of the insulin receptor substrate-1. This blocked insulin-dependent tyrosine autophosphorylation of the insulin receptor and/or tyrosine phosphorylation of insulin receptor substrate-1, and attenuated insulin action in 3T3-L1 adipocytes, 32D myeloid cells and Fao hepatoma cells [190–192]. Further, evidence suggests that, in L6 rat skeletal muscle cells, TNF $\alpha$ , via ceramide, inhibited protein phosphatase-1 activation, thereby attenuating insulin-stimulated glucose uptake, glycogen synthase activity and glycogen synthesis [126]. Further, in 3T3-L1 adipocytes, ceramide mimicked the effect of TNF $\alpha$  in down-regulating the glucose transporter GLUT4 and attenuating glucose uptake [193].

Ceramide signalling may also predispose to diabetes by affecting pancreatic  $\beta$ -cell function. In the pancreas, macrophages infiltrating the islet cells produce IL-1 $\beta$ , which is believed to play a role in triggering the onset of type 1 diabetes [195]. Ceramide mimicked the effects of IL-1 $\beta$  on this system, causing inhibition of insulin production [196], perhaps by activating a protein phosphatase 2A [197]. Unger and co-workers proposed another mechanism by which ceramide might initiate type 1 diabetes [198]. These investigators showed that islets from Zucker fatty diabetic rats manifested elevated ceramide levels and apoptosis. In response to a challenge with non-esterified fatty acids, these islets displayed reduced fatty acid oxidation and markedly increased incorporation into ceramide, accompanied by apoptosis. FB1 blocked both ceramide generation and apoptosis, indicating that ceramide generation was necessary for the apoptotic response. Thus ceramide may be involved in induction of a pro-diabetic state via regulation of insulin action and/or the bioavailability of insulin.

In the ovary, TNF $\alpha$ , released by macrophages, oocytes and other follicular cells, may provide a physiological stimulus for ceramide generation and apoptosis of granulosa cells during follicle atresia [199]. Similarly, Hsueh and co-workers showed that ceramide, like TNF $\alpha$ , antagonized the effects of follicle-stimulating hormone in isolated follicles, inducing apoptosis of follicular cells [138]. Fanjul and co-workers have also proposed

that, in gonadotrophin-stimulated granulosa cells, ceramide mediates IL-1 $\beta$  inhibition of progesterone synthesis [200]. In these cells, ceramide stimulated transcription of cyclo-oxygenase and prostaglandin endoperoxide synthase, enzymes involved in prostaglandin E<sub>2</sub> synthesis [200]. Further, in cells primed with follicle-stimulating hormone, ceramide mimicked TNF $\alpha$  inhibition of aromatase, an enzyme involved in oestrogen biosynthesis [201]. Hence ceramide regulates a number of endocrine functions in different cell types by serving as a second messenger for cytokine function.

### Vascular system

In the vascular system, ceramide regulates both apoptosis and inflammation. SMase activation and ceramide generation in response to cytokines or environmental stress stimuli have been implicated in the pathogenesis of several diseases, including radiation pneumonitis [81,202], septic shock [203], atherogenesis [16,17,204,205] and ischaemic heart disease [206].

Several reports suggest that ceramide mediates apoptosis in BAEC and human vascular endothelial cells in response to stresses such as radiation (see below), TNF $\alpha$ , oxidative stress and heat shock [49,81,207]. In addition, recent investigations by Haimovitz-Friedman et al. suggest that ceramide also plays a role in the pathogenesis of endotoxic shock, by acting as a mediator of disseminated endothelial apoptosis [203]. In the endotoxic shock syndrome, the bacterial lipid LPS initiates, via host TNF $\alpha$  and other cytokines, systemic inflammation, endothelial cell damage and intravascular coagulopathy [208]. Death results from extensive tissue injury, multiple organ failure and circulatory collapse. Injection of LPS or TNF $\alpha$  into C57/BL6 mice induced microvessel endothelial apoptosis in numerous tissues, which occurred prior to non-endothelial damage, and was preceded by tissue generation of ceramide. TNF $\alpha$  was required for these events, since TNF-binding protein, which protects against LPS-induced death, blocked LPS-induced ceramide generation and endothelial apoptosis. Acid SMase knock-out mice displayed a normal increase in serum TNF $\alpha$  in response to LPS, yet were protected in part against endothelial apoptosis and death, implicating ceramide as one mediator of the endotoxic response. Further, intravenous injection of bFGF, which acts as an intravascular survival factor for endothelial cells [209], blocked LPS-induced ceramide elevation, endothelial apoptosis and animal death, but did not affect the LPS-induced elevation of serum TNF $\alpha$ . These investigations demonstrate that LPS induces a disseminated form of endothelial apoptosis, mediated sequentially by TNF $\alpha$  and ceramide generation, and suggest that this cascade is critical to the development of endotoxic shock syndrome.

Ceramide has also been implicated in other aspects of TNF $\alpha$  action on endothelial cells. Treatment of human umbilical vein cells with low doses of SMase increased signalling through the MAPK cascade, whereas high doses induced expression of the inflammatory cytokines IL-6 and IL-8, and of the adhesion molecules E-selectin, VCAM and ICAM, which mediate neutrophil adherence to the endothelium [122]. In another study, treatment of these cells with a synthetic ceramide analogue increased IL-1 $\beta$ -induced E-selectin transcription and expression, suggesting that ceramide may act as a co-signal for inflammatory mediators in endothelial cells [210]. Exposure of human umbilical vein cells to SMase or ceramide analogues also increased the release of plasminogen activator inhibitor-1, which inhibits the thrombolytic activity of tissue plasminogen activator [211]. Thus, while ceramide mediates endothelial apoptosis in certain circumstances, in others it signals inflammation.

Ceramide may contribute to the development of atherosclerotic or thrombotic disease. Atherosclerosis is a disease process characterized by aggregation of lipoproteins in the arterial wall, foam cell formation and smooth muscle cell proliferation [212]. Retention of low-density lipoprotein (LDL) within the arterial wall, which may be enhanced by aggregation of LDL particles, appears to be a critical event mediating development and progression of the atherosclerotic lesion [212]. Tabas and co-workers showed that exogenous SMase and lipoprotein lipase synergized to induce a 10–100-fold increase in LDL aggregation on bovine aortic smooth muscle cells exposed to LDL [213]. An arterial wall SMase activity, perhaps secreted by endothelium or macrophages [16], similarly initiated LDL aggregation via generation of ceramide in the LDL particles [204]. Further, co-incubation of aggregated LDL-bearing smooth muscle cells with macrophages induced the macrophages to phagocytose the aggregates and attain the foam cell morphology typical of atherosclerotic lesions. Ceramide generation might also mediate smooth muscle cell proliferation. Exposure of these cells to oxidized LDL resulted in rapid SMase activation and ceramide generation which correlated with cell proliferation [205,214]. Further, exogenous ceramide mimicked this mitogenic effect. Perhaps most importantly, aggregated lesional LDL is 10–50-fold enriched in ceramide compared with plasma LDL [204].

Exogenous ceramide and SMase cause relaxation of rat thoracic aortic rings treated with the vasoconstrictor phenylephrine. Hence it was proposed that cytokine-induced SMase activation and ceramide generation may signal vasodilatation [215]. In cardiac myocytes, TNF $\alpha$  treatment induced rapid SM hydrolysis and ceramide generation, followed by an increase in free sphingosine. Exogenous sphingosine mimicked the negative inotropic effects of TNF $\alpha$  in contracting myocytes, and inhibition of sphingosine production with the ceramidase inhibitor *N*-oleoylethanolamine blocked the negative effects of TNF $\alpha$  on myocyte contractility [216].

Ischaemia/reperfusion injury is accompanied by the production of TNF $\alpha$  and generation of free radicals. Rapid changes in ceramide and sphingosine levels have been detected, as well as activation of JNK, but not of MAPK. In a mouse model of renal ischaemia/reperfusion, it was found that ceramide and sphingosine levels dropped during ischaemia and that, during reperfusion, ceramide levels almost doubled compared with controls. Thus ceramide may contribute to post-ischaemic acute renal failure [217], perhaps via activation of JNK, which signals TNF $\alpha$  transcription [218,219].

Hence ceramide affects endothelial cells, smooth muscle cells and cardiomyocytes of the cardiovascular system. Depending on the initiating stimulus or insult, ceramide may cause apoptosis, regulate a variety of differentiated functions or contribute to the development of pathological states such as endotoxic shock, atherosclerosis or ischaemia/reperfusion injury.

### Central nervous system

Perhaps the most compelling work concerning the role of ceramide in signalling in the central nervous system centres on neurotrophin activation of the p75 receptor (p75<sup>NTR</sup>), a member of the TNF receptor superfamily [220]. Neurotrophins are a class of growth factors which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-5. Neurotrophins interact with two classes of cell surface receptors, the p75<sup>NTR</sup> and the Trk tyrosine kinase receptors. Dobrowsky et al. showed that binding of neurotrophins to p75<sup>NTR</sup> in cells lacking Trk receptors [221] causes SM hydrolysis to ceramide, perhaps in caveolae [11]. The cellular

response to p75<sup>NTR</sup>-mediated ceramide generation is variable, depending on cell type and co-ordinate signalling through Trk receptors. In most instances, activation of p75<sup>NTR</sup> in the absence of a strong Trk signal induces apoptosis or impairs differentiated function [222,223]. To study p75<sup>NTR</sup> in isolation, for example, normal glial cultures and several gliomas have been used that express p75<sup>NTR</sup> and TrkB, but not TrkA. Since NGF does not activate TrkB, addition of NGF to these cells stimulates only p75<sup>NTR</sup> [224]. Consequently, in T9 glioma cells, evidence suggests that ceramide, generated by NGF activation of p75<sup>NTR</sup>, mediates growth inhibition [225], whereas in primary neonatal rat oligodendrocytes, ceramide appears to signal JNK activation and apoptosis when NGF is applied [226,227]. Similar results were obtained with TNF $\alpha$  in mature human oligodendrocytes [228]. Ceramide-mediated apoptosis in oligodendrocytes, and in other cells of the central nervous system, involves caspase activation [131,137,229]. Through TNF $\alpha$  and/or CD95, ceramide-induced apoptosis may play a role in experimental allergic encephalomyelitis [226,230] or multiple sclerosis [231–233], demyelinating syndromes involving oligodendrocytes, infiltrating lymphocytes and microglia.

In models in which both p75<sup>NTR</sup> and the Trk receptors are studied together, the complexity increases. When TrkA is transfected into oligodendrocytes, application of NGF can then stimulate p75<sup>NTR</sup> and TrkA together, which results in suppression of JNK activation and cell death [234]. Analysis of TrkA mutants in fibroblasts revealed that TrkA exerts anti-apoptotic effects by activating the MAPK and Akt/protein kinase B kinase cascades [235], which may inhibit SMase signalling [221]. In a somewhat analogous situation with dopaminergic mesencephalic neurons, NGF can stimulate only p75<sup>NTR</sup>, resulting in ceramide generation and secretion, but BDNF stimulates both p75<sup>NTR</sup> and TrkB together, in which case ceramide signalling is suppressed [236]. Evidence has emerged for at least one mechanism by which p75<sup>NTR</sup>, through ceramide, may transmodulate Trk function. In PC12 cells that normally lack TrkB, BDNF selectively engages p75<sup>NTR</sup> and, via ceramide, attenuates TrkA activity by promoting serine phosphorylation of TrkA [237]. Additional mechanisms for the phenotype-specific differences in ceramide and Trk interaction require identification.

A body of work is emerging on the role of ceramide signalling in central nervous system cells independent of neurotrophin receptor activation. In neurons, multiple and at times opposing outcomes have been attributed to ceramide generation. Based on *in vitro* studies with primary neuronal cultures, a neuroprotective role for ceramide has been suggested in Alzheimer's disease and Parkinson's disease [160,238,239]. Primary sympathetic neurons undergo apoptosis upon neurotrophin withdrawal, and exogenous ceramide prevents this [240]. C<sub>2</sub>-ceramide also protects cultured rat hippocampal neurons from death induced by amyloid  $\beta$ -peptide and excitotoxic stress such as glutamate [160,241]. For hippocampal neurons exposed to oxidative stress, evidence suggests that ceramide-induced NF- $\kappa$ B activation mediates the neuroprotection afforded by TNF $\alpha$  [241–243].

Additional experiments with primary hippocampal neuronal cultures indicated more complex effects of ceramide; it initially promotes neurite extension [244], but later (during the second to third day), unless converted into glucosylceramide, prolonged ceramide elevation induces apoptosis. Further, during NGF-induced PC12 cell differentiation, exogenous ceramide impaired TrkA-mediated neurite extension [245], in part by stimulating cleavage of microtubule-associated tau protein by calpain I [246]. Other examples of neurotoxicity can be found. Dopaminergic neurons in primary cultures of the mesencephalon, an important region of neuronal degeneration in Parkinson's disease, and

cerebellar granule neurons also undergo apoptosis in response to  $C_2$ - and  $C_6$ -ceramide and oxidative stress [247,248]. Further, staurosporine, a kinase inhibitor which mimics some of the neuroprotective effects of NGF *in vivo* [249], causes SM hydrolysis to ceramide in embryonic chick cortical neurons *in vitro*, and apoptosis [250,251]. Elevation of cellular ceramide levels with  $C_2$ -ceramide, exogenous SMase or the ceramidase inhibitor *N*-oleoylethanolamine similarly induced apoptosis in these cells, as well as in immortalized hippocampal neurons and dorsal root ganglion cells. These apparently bipotential or contradictory results of ceramide treatment may reflect ceramide concentration and/or the state of cellular differentiation. In this regard, Mitoma et al. [252] showed that doses of up to 3  $\mu$ M ceramide promoted growth and differentiation of immature hippocampal neurons, while doses above 10  $\mu$ M induced apoptosis. In mature neurons, however, ceramide induced cell death at all doses.

Ceramide, like JNK [253], has been associated with tissue damage during ischaemia and stroke. Kubota et al. demonstrated SM breakdown and ceramide generation at the site of arterial occlusion in the brain [254–256]. Whether this ceramide elevation is neuroprotective or induces oligodendrocyte damage is uncertain. As in other systems, JNK activation by stress and ceramide is often [131,226,228,229,248,257], but not always [226–228,257,258], pro-apoptotic.

Death and survival are not the only end-points of ceramide signalling in the central nervous system. Ceramide affects inter-cellular communication by stimulating the release of factors such as dopamine [236], IL-6 and leukaemia inhibitory factor [259,260]. In astrocytes, pro-inflammatory cytokines, via ceramide, signal NGF [261] and nitric oxide [262] production through Raf/Ras/MAPK. Ceramide also induces cytoplasmic translocation and activation of PKC $\zeta$  in astrocytes [263,264]. In oligodendrocytes, TNF $\alpha$  and SMase rapidly inhibited myelin basic protein and 2',3'-cyclic nucleotide 3'-phosphodiesterase phosphorylation [265,266], attenuating growth and differentiation. Ceramide mimicked the synergistic effect of TNF $\alpha$  on IFN $\gamma$ -induced inhibition of cytomegalovirus viral replication in astrocytoma cells [267]. In contrast, TNF $\alpha$  through ceramide may enhance HIV infection [268]. In astrocytes, the HIV-1 *nef* gene product enhanced TNF-induced ceramide generation and NF- $\kappa$ B activation, while blocking AP-1 activation [269], perhaps shifting cells towards survival and contributing to the gliosis characteristic of AIDS encephalopathy.

While central nervous system cells have properties unique to this system, the accumulating data show that ceramide signalling in the central nervous system has much in common with that in other systems.

## ROLE OF CERAMIDE IN RADIATION- AND DRUG-INDUCED CELL DEATH

The most widely accepted mechanism of ionizing-radiation-induced cell death involves the nucleus as the primary target for damage and DNA double-strand breaks as the causative lesions. Genetic instability associated with unrepaired or misrepaired DNA breaks results in progeny cell death after several mitotic cycles [270–272]. Hence this form of cell death is called mitosis-dependent or clonogenic cell death. However, in endothelial, thymic, lymphoid, haematopoietic and certain stem cell types, radiation kills cells via apoptosis. This form of cell death occurs mainly in cells arrested in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle, and hence is an interphase form of cell death.

Recent investigations have implicated ceramide as a mediator of radiation-induced apoptosis. BAEC, U937, BL30A Burkitt's lymphoma, WEHI-231B, HL-60 and TF-1 erythromyeloblastic

cells exposed to therapeutic doses of ionizing radiation were shown to undergo rapid SM hydrolysis, ceramide generation and apoptosis [52,207,273,274]. Radiation also induced rapid activation of SMase in a nuclear-free cell lysate, indicating a direct effect of radiation on the membrane, independent of the effect on nuclei. Further, lymphoblasts from NP patients were defective in radiation-induced ceramide generation and apoptosis [81]. Restoration of the SMase activity by retroviral transfer of human acid SMase cDNA restored both events. To test further the role of acid SMase in the apoptotic response to radiation, knock-out mice generated by targeted disruption of the acid SMase gene in exon 2 were used [81]. The homozygous knock-out animals develop normally until adulthood, and then begin to manifest the neurological abnormalities characteristic of NP disease. Acid SMase knock-out mice exposed to as much as 20–30 Gy of ionizing radiation, 3–4 weeks after birth, failed to generate a ceramide elevation or an apoptotic response in the lung endothelium, whereas control animals manifested extensive pulmonary endothelial apoptosis. In other tissues such as the thymus, SMase did not appear to be required for radiation-induced apoptosis, as the SMase knock-out mice were not protected. In contrast, in p53 knock-out mice, the thymus was protected from radiation-induced apoptosis, but the lung was not. These studies show that a single stress can signal apoptosis by different mechanisms in different tissues.

Additional support for the role of ceramide in radiation-induced apoptosis comes from the work of Weichselbaum and co-workers [273,275,276]. This group reported that cells deficient in ceramide production, selected by use of *N*-oleoylethanolamine, were resistant to radiation-induced apoptosis, while de-repressing SMase using PKC inhibitors enhanced radiation sensitivity. Further, Debatin and colleagues [50] have proposed that ceramide, generated in response to  $\gamma$ -radiation and chemotherapy, up-regulated expression of the CD95/Apo-1/Fas ligand (CD95-L), possibly via a mechanism requiring activation of JNK [277], and that the binding of the ligand to CD95 mediated the therapeutic response to these agents. Consistent with this proposal, fibroblasts from NP patients failed to up-regulate CD95-L expression and undergo apoptosis after irradiation or exposure to chemotherapeutic drugs, but addition of exogenous ceramide restored CD95-L expression and apoptosis.

Ceramide has been implicated in the apoptotic response to several chemotherapeutic drugs. Treatment of P388 T cells or U937 cells with daunorubicin results in apoptosis, which is preceded by ceramide generation via activation of the enzyme ceramide synthase [26], as described above. Alternatively, daunorubicin-induced ceramide generation might, in some instances, result from neutral SMase activation [44]. Ceramide generation has also been shown to be involved in vincristine-induced apoptosis in ALL-697 cells and in 1- $\beta$ -D-arabinofuranosylcytosine (ara-C)-induced apoptosis in Jurkat, EL-4 and HL-60 cells [53–55].

Studies have shown that, in radiation- and drug-induced apoptosis, the pro-apoptotic ceramide signal is counter-balanced, in certain cells, by an anti-apoptotic signal mediated via DAG [278]. In this context, several studies have shown that radiation activates PKC $\alpha$  via DAG generation [279–281]. Further, in several different cell types, activation of PKC by phorbol esters blocks radiation-induced ceramide generation and apoptosis [207,273,282]. In addition, pharmacological inhibition of PKC enhanced radiation-induced cell death [283,284], while exogenous DAG or phorbol ester blocked radiation- or ceramide-induced apoptosis [207,285]. Thus PKC blocks both ceramide generation and action during induction of apoptosis. These protective effects of PKC may occur via inhibition of SMase [207], or in some cells

by up-regulation of Bcl-2 or Bcl-X<sub>L</sub> [286,287]. The endothelial survival factor bFGF also protected endothelial cells against radiation-induced apoptosis, in part via activation of the PKC and MAPK pathways [209,288]. In chemotherapeutic drug-induced apoptosis, a similar control mechanism exists. Daniel and co-workers have reported that, in ara-C treated HL-60 cells, both ceramide and DAG generation are stimulated. While exogenous ceramide mimicked ara-C-induced apoptosis, exogenous DAG or phorbol ester blocked apoptosis, and inhibition of PKC $\beta$  enhanced ara-C-induced apoptosis [53,55,289]. Thus ceramide and DAG appear to generate antagonistic signals that determine cell survival or death after exposure to chemotherapy or radiation.

## CONCLUSIONS

Ceramide has been shown to affect almost every cell or tissue type examined, causing a wide range of effects in a cell-type-specific and context-dependent manner. In several instances, stimuli that lead to ceramide generation also cause DAG generation or SIP formation, and these molecules block the effects of ceramide. This high level of regulation may provide opportunities for pharmacological intervention. In the treatment of cancer, suppression of anti-apoptotic signals might increase the sensitivity of target tissues to radiation and chemotherapy. Alternatively, activation of anti-apoptotic signals could prevent the inadvertent death of normal tissue surrounding tumours. Inhibition of ceramide signalling may also provide protection from endotoxic shock, and attenuate ceramide-mediated effects in ischaemia/reperfusion injury, in the development of atherosclerosis and in insulin-resistant diabetes. An understanding of the mechanisms by which ceramide regulates physiological and pathological events in specific cells may provide new targets for therapeutic intervention.

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