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COMMENTARY

Stress-Induced Apoptosis and the Sphingomyelin Pathway

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ABSTRACT. The sphingomyelin pathway is a ubiquitous, evolutionarily conserved signaling system initiated by hydrolysis of the plasma membrane phospholipid sphingomyelin to generate the second messenger ceramide. Sphingomyelin degradation is catalyzed by acid and neutral sphingomyelinase (SMase) isoforms. Most, if not all mammalian cells, appear capable of signaling though the sphingomyelin pathway. Diverse receptor types and environmental stresses utilize the sphingomyelin pathway as a downstream effector system. In some cellular systems, ceramide initiates differentiation or cell proliferation, while in other systems, ceramide signals apoptosis. Recent investigations link the activation of neutral SMase to the extracellular signal regulated kinase (ERK) cascade and pro-inflammatory responses, and acid SMase to the stress-activated protein kinase/c-jun kinase (SAPK/JNK) cascade and apoptotic responses. Environmental stresses act directly on membrane to activate acid pH-dependent sphingomyelinase (ASMase), whereas cytokine receptors signal ASMase activation through motifs termed death domains. The present review focuses on mechanisms of activation of ASMase and on ceramide signaling of the apoptotic response. BIOCHEM PHARMACOL 53;5:615–621, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. apoptosis; sphingomyelin; sphingomyelinase; ceramide; ICE proteases; environmental stress; cytokines

The ability of organisms to eliminate selected types of cells by apoptosis (programmed cell death) represents a major regulatory mechanism in development, growth, and differentiation. The biochemical pathways of apoptosis can be triggered by a variety of physiologic stimuli and environmental stresses. Tightly regulated cascades of overlapping or competing cellular signals effect the activation of specific proteases and nucleases that carry out an ordered disassembly of cellular structures. The regulation of these signal transduction cascades is currently the subject of intensive investigation. Inhibition of apoptosis or disruption of the balance between growth and apoptotic signals is implicated in autoimmune disorders and cancer, while increased apoptosis is believed to be associated with degenerative diseases [1, 2]. It is, therefore, suggested that advances in the understanding of the biochemical events involved in these processes may lead to the development of selective pharmacological interventions to improve the outcome in treatment of human diseases.

Whereas the biochemical apparatus of the apoptotic pathway remains to a large extent unknown, it is generally accepted that in mammalian cells apoptosis is effected by the interleukin 1β-converting enzyme (ICE§) family of proteases [3, 4]. These enzymes can be classified functionally into two groups, ICE-like and ced-3-like proteases, based on their ability to recognize and cleave substrates containing the sequences -YVAD- or -DEVD-, respectively. This property allows synthetic tetrapeptides with corresponding sequences to serve as competitive inhibitors of ICE/ced-3 enzyme action *in vitro* and *in vivo*. The ICE/ced-3 proteases are expressed in the intact cell in the form of zymogens, activated either by autocatalysis, or by cleavage by other ICE/ced-3 proteases or the serine protease granzyme B. By analogy to other protease cascades, it has been suggested that a hierarchy of trans-cleavage of ICE/ced-3-related proteases controls the orderly progression of the apoptotic disassembly of cellular organelles. Ectopic expression of ICE in mammalian cells results in apoptosis [5] and ICE-like proteases also have been shown to be responsible for apoptosis in a cell-free system [6]. Recent studies show that Fas/Apo-1/CD95-induced apoptosis requires activation of an ICE-like-protease upstream of a ced-3 CPP32/Apopain/Yama-like protease [7–9].

A great deal of attention has been directed recently at

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§ *Abbreviations:* ICE, interleukin 1β-converting enzyme; ASMase, acid pH-dependent sphingomyelinase; NSMase, neutral pH-dependent sphingomyelinase; ERK, extracellular signal regulated kinase; SAPK, stress-activated protein kinase; JNK, c-jun kinase; TNF, tumor necrosis factor; NPD, Niemann-Pick disease; NSD, NSMase activating domain; FAN, factor activating NSMase; CAPK, ceramide-activated protein kinase; CAPP, ceramide-activated protein phosphatase; PKC, protein kinase C; SPP, sphingosine 1-phosphate; TRADD, TNF receptor 1-associated death domain; FADD, Fas-associated death domain protein; MORT1, mediator of receptor-induced cytotoxicity; and DED, death effector domain.

mechanisms by which extracellular signals activate the ICE/ced-3 proteases to effect apoptosis. Two distinct transmembrane signaling systems have been characterized: the sphingomyelin pathway, which utilizes turnover of membrane sphingolipids to generate the second messenger ceramide and may be involved in cytokine- and environmental stress-induced apoptosis, and a "death domain" adapter protein system, which specifically mediates the apoptotic function of cytokine receptors, such as TNF-α and Fas/Apo-1/CD95. This review will focus on the role of the sphingomyelin pathway in stress-induced apoptosis, and a possible mode of integration of the two effector systems will be explored.

THE SPHINGOMYELIN-CERAMIDE PATHWAY

Ceramide serves as a second messenger of the sphingomyelin pathway (Fig. 1), stimulating specific kinases, phosphatases, and transcription factors that mediate a variety of cellular functions [for reviews, see Refs. 10–13]. Ceramide is the backbone of all sphingolipids and glycosphingolipids and, thus, is subject to complex metabolic regulation. However, within the context of signal transduction, ceramide can be generated by two mechanisms. It can be synthesized by condensation of the sphingoid base sphinganine and fatty acyl-CoA by the enzyme ceramide synthase (EC 2.3.1.24, sphinganine N-acyl transferase) to form dihydroceramide, followed by a rapid oxidation to ceramide. This route has been shown to be involved in daunorubicin-induced apoptosis [14]. An alternative and more prevalent system for ceramide generation involves the degradation of sphingomyelin into phosphorylcholine and ceramide by the action of sphingomyelin-specific forms of phospholipase C, termed sphingomyelinases (EC 3.1.4.12, sphingomyelin phosphodiesterase).

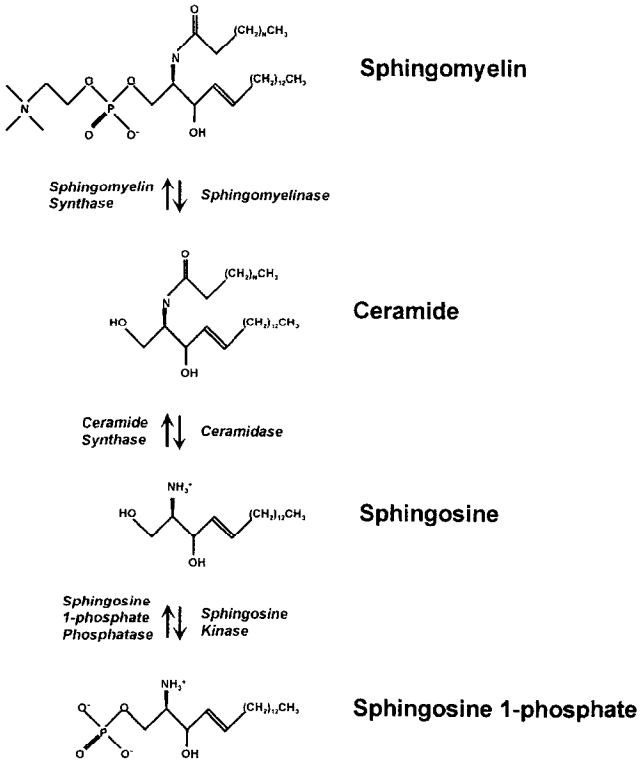

FIG. 1. Sphingomyelin metabolites involved in signaling.

The sphingomyelin pathway is a ubiquitous, evolutionarily conserved signaling system analogous to the phosphoinositide pathway. Similar to the action of diacylglycerol, generated by a phosphoinositide-specific phospholipase C, ceramide can initiate a variety of cellular effects. In some cellular systems, ceramide has been reported to initiate differentiation [15] or cell proliferation [16–18], while in other systems, ceramide signals apoptosis. Activation of sphingomyelinases has been linked to several cell surface receptors, such as the 55 kDa TNF-α receptor [19–22], the 80 kDa interleukin-1 receptor [23, 24], the 75 kDa neurotrophin receptor ($p75^{NTR}$) [25, 26], and CD95 [27, 28], to list a few. Further, numerous stresses that initiate apoptosis have also been associated with rapid ceramide generation via sphingomyelinase activation, including ionizing radiation, ultraviolet-C, heat shock, oxidative stress, daunorubicin, and vincristine [14, 22, 29–33]. Cell-permeable ceramide analogs have been useful agents in studying signaling through the sphingomyelin pathway [12, 13]. In this regard, ceramide analogs, but not analogs of other lipid second messengers, mimic the effect of cytokines and environmental stresses.

Ceramide generation via sphingomyelinase activation has been shown to involve either an ASMase or an NSMase, which exists as Mg^{2+}-dependent or -independent forms [34]. Human and murine ASMases have been cloned and determined to be the products of single genes, while NSMase has yet to be characterized at the molecular level. However, ASMase knockout (ASM-KO) mice retain NSMase activity, indicating that NSMase is the product of a distinct gene or genes [35, 36].

Krönke and co-workers have explored the mechanisms of activation of the ASMase and NSMase by mutational analysis of the 55 kDa TNF receptor [21, 37]. The cytoplasmic portion of the TNF receptor contains two distinct regions that differentially associate with ASMase or NSMase signaling (Fig. 2). A membrane proximal region comprised of an 11 amino acid motif termed the NSD is specifically associated with NSMase signaling of proinflammatory cellular responses [37]. A novel adaptor protein that has been identified recently, termed FAN, binds to the TNF receptor NSD motif promoting NSMase activation [39]. In contrast, a membrane distal region of the cytoplasmic domain of the TNF receptor links to the ASMase [21]. This region comprises a 75 amino acid motif, termed the "death domain" [40]. Deletions or mutations in the death domain abolish TNF receptor-induced apoptosis. The death domain is conserved in CD95, a TNF receptor homolog that mediates apoptosis in lymphocytes and other CD95-expressing cells. Mutations within the death domain, which abolish CD95-induced apoptosis, also block

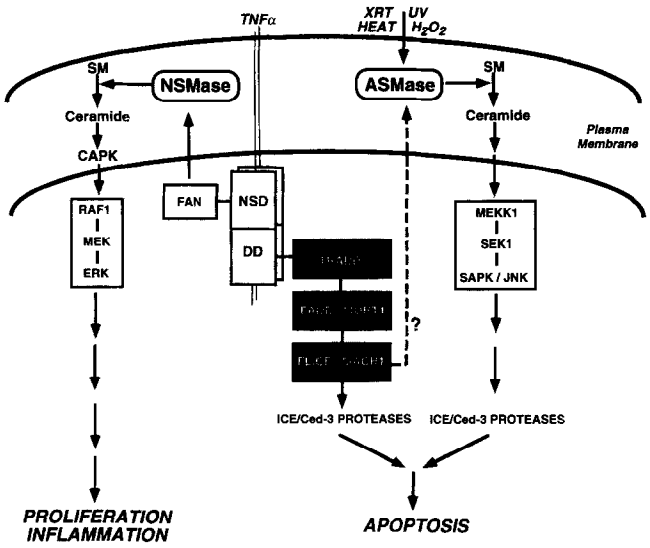

FIG. 2. Proposed mechanism for stress and TNF receptor signaling via the sphingomyelin pathway. Environmental stresses act directly upon membrane and activate ASMase, generating ceramide and initiating signaling through the SAPK/JNK cascade. It is presumed that SAPK/JNK phosphorylates a target upstream of the ICE/ced-3 proteases that effect apoptosis. The 55 kDa TNF receptor delivers proliferative/pro-inflammatory and apoptotic signals through the sphingomyelin pathway. A membrane proximal region of the cytoplasmic domain of the TNF receptor links through the adaptor protein FAN to a NSMase isoform catalyzing sphingomyelin hydrolysis to ceramide in the plasma membrane. Ceramide then stimulates CAPK, which phosphorylates and activates Raf-1, and the ERK cascade. In cells in which TNF initiates apoptosis, TNF–TNF receptor interaction results in formation of a multi-protein complex that links the death domain (DD) in the membrane distal region of the cytoplasmic domain of the receptor to the adaptor proteins TRADD, FADD/MORT1, and pro-FLICE/MACH1. Autocatalysis of FLICE/MACH1 is presumed to initiate a cascade of ICE/ced-3 proteases. The DD region also links to ASMase, perhaps via a phosphatidylcholine-specific phospholipase C (PC-PLC) [38]. Deletions of the DD region of the TNF receptor [21] and CD95 [28], as well as overexpression of dominant negative FADD/MORT1 [8], block ligand-induced ceramide generation but not apoptosis initiated by ceramide analogs. Hence, ASMase activation can be molecularly ordered downstream of the DD adaptor protein complex. As for environmental stress, ceramide generated via this mechanism eventually signals through the SAPK/JNK cascade. It is presumed that the effects of ceramide act in concert with ICE/ced-3 proteases activated directly through FLICE/MACH1 to mediate apoptosis.

activation of ASMase [28]. Whether the linkage between the death domains and ceramide is a general mechanism for the induction of apoptosis, and the molecular ordering of this putative pathway, remain unknown.

A substantive body of evidence now exists defining ceramide as a message for the induction of apoptosis. In intact cells, rapid ceramide generation is an early event in the apoptotic response to numerous stimuli including cytokines and environmental stresses, and ceramide analogs mimic the effect of stress and induce apoptosis [10–13]. More substantial confirmation comes from "cell-free" studies using membrane fractions devoid of nuclei. Membranes isolated from cells treated with the cytokine TNF-α or subjected to the environmental stress of ionizing radiation exhibit ceramide generation via sphingomyelinase activation [20, 29], and can initiate nuclear apoptosis when combined with cytosol and nuclei from untreated cells (Mathias S, Billis W and Kolesnick R, unpublished observations). More importantly, ceramide analogs, when added directly to the cell-free system, can also initiate the apoptotic program [31]. These latter data are consistent with activation by ceramide of a pre-programmed signaling pathway for induction of apoptosis.

The most convincing evidence that ceramide is critical for induction of apoptosis is derived from studies using two genetic models of ASMase deficiency. Lymphoblasts from patients with NPD [41], an inherited deficiency of ASMase, and from transgenic ASM-KO mice [35, 36] manifest defects in the apoptotic response [42]. NPD lymphoblasts fail to respond to ionizing radiation with ceramide generation and apoptosis, whereas normal cells generate ceramide and are killed. These abnormalities were reversible in NPD lymphoblasts upon restoration of ASMase activity by retroviral transduction of human ASMase cDNA [42]. Similarly, ASM-KO mice failed to exhibit the apoptotic response to radiation observed in several types of normal tissues. When compared with the p53 knockout mouse, the patterns of defects in the apoptotic response were markedly different. In some tissues such as the endothelium of the lung and heart, and the mesothelium of the pleura and pericardium, radiation-induced apoptosis appears primarily dependent on ASMase and, for the most part, independent of p53. In contrast, thymic apoptosis appears highly dependent on p53 and, for the most part, independent of ASMase. Hence, radiation appears capable of activating two apparently independent signaling mechanisms for induction of apoptosis. These genetic models provide definitive evidence for the involvement of ASMase in at least one form of stress-induced apoptosis.

Although the immediate target for ceramide in induction of the apoptotic response is at present uncertain, a number of direct cellular ceramide targets have been defined. One target is a proline-directed, serine/threonine-specific ceramide-activated protein kinase (CAPK) [43, 44]. CAPK phosphorylates and activates Raf-1 [45], and is thought to mediate proliferative and pro-inflammatory responses to TNF-α via the ERK cascade. For several years, a ceramide-activated protein phosphatase (CAPP) with PP2A activity (serine/threonine specific) has been known [46], but molecular characterization of CAPP was achieved only recently after genetic screening of yeast mutants resistant to ceramide inhibition of growth [47]. Yeast CAPP is a heterotrimer of previously known proteins, consisting of two regulatory subunits and a catalytic subunit. Ceramide apparently activates the B subunit (Cdc55p), whereas the A subunit (Tpd3p) is required to bring the B subunit into association with the catalytic C subunit (Sit4p). Other tar-

gets of ceramide activation include an isoform of protein kinase C, PKC-ζ, which is coordinately regulated by ceramide and arachidonic acid [48, 49], and the putative guanine-nucleotide exchange factor Vav [50]. Which, if any, of these ceramide targets initiate the apoptotic response is presently an area of active investigation.

CERAMIDE AND THE SAPK/JNK PATHWAY

Protein kinase cascades commonly serve as downstream effector systems linking cell surface receptors to cellular activation via second messengers. In particular, mitogen-activated protein (MAP) kinase cascades have been associated recently with apoptosis. These cascades are comprised of sets of protein kinases that are sequentially phosphorylated and activated, resulting in transmission of the signal from the cell surface through the cytoplasm (Fig. 2). Eventually, cell-type specific substrates are targeted. The ERK cascade has been shown to communicate growth factor- and phorbol ester-induced proliferative signals. Recently, stress-activated protein kinase or SAPK (also known as c-Jun kinase, or JNK), and p38 cascades have been defined. These cascades, activated by environmental stresses such as inflammatory cytokines, ATP depletion, heat and osmotic shock, ionizing and UV irradiation, and endotoxin have been shown to transmit growth arrest, differentiation, or apoptotic signals [51–53].

Recent evidence links ceramide-induced apoptosis to the SAPK/JNK protein kinase cascade in a variety of cell types. Verheij *et al.* [32] reported that ionizing radiation, ultraviolet C radiation, H_2O_2, heat shock, and TNF-α induce ceramide generation within seconds in primary cultures of bovine endothelial cells and U937 monoblastic leukemia cells, prior to activating the SAPK/JNK pathway. Further, a number of studies showed that ceramide analogs, like stress, activated the SAPK/JNK cascade [32, 54–57]. Disruption of signaling down the SAPK/JNK cascade but not the ERK cascade, by overexpression of dominant negative mutants, abrogated TNF-, stress- and ceramide-induced apoptosis. These studies provided evidence that the SAPK/JNK cascade was a downstream effector system for induction of apoptosis via ceramide.

In contrast, ceramide and stress result in little or no change in ERK pathway activation in cells undergoing apoptosis [32, 56, 57]. In fact, evidence has been provided that the ERK cascade is anti-apoptotic. Greenberg and co-workers [58] showed that withdrawal of nerve growth factor from PC-12 cells resulted in activation of both SAPK/JNK and p38 signaling systems coordinated with inactivation of ERK, and apoptosis. Using dominant-interfering or constitutively activated forms of these enzymes, it was demonstrated that the inactivation of the ERK cascade was required in addition to activation of the SAPK/JNK and p38 cascades for apoptosis to proceed in these cells. This suggests that a balance between SAPK/JNK and ERK may be critical in effecting the apoptotic outcome.

Taken together, these data may suggest a mechanism by which the SAPK/JNK signaling system may link ceramide signaling at the cell surface through to ICE/ced-3 proteases to effect the apoptotic response. Consistent with this hypothesis, Hannun and co-workers recently reported that ceramide-initiated apoptosis was mediated by a CPP32-like protease, which was inhibitable by Bcl-2 [33, 59]. Cells overexpressing Bcl-2, like wild-type cells, still responded to vincristine treatment with ceramide generation, but failed to show CPP32 activation and apoptosis in response to vincristine or a C6-ceramide analog.

The concept that SAPK/JNK signaling might be obligatory for TNF-induced apoptosis was addressed in recent studies by Spiegel and co-workers [55]. Treatment of U937 monoblastic leukemia cells with TNF-α or ceramide analogs induced SAPK/JNK activation and apoptosis, while SPP (Fig. 1), a lipid second messenger associated with calcium mobilization, activated the ERK cascade and stimulated proliferation. Addition of SPP concomitant with TNF or ceramide analogs blocked signaling through the SAPK/JNK cascade for both agonists, and inhibited apoptosis. However, SPP did not affect TNF-induced ceramide generation. The fact that SPP inhibited the apoptotic response to TNF suggests either that transmodulatory inactivation of SAPK/JNK blocks apoptosis, or that SPP interferes with the assembly or function of the TNF receptor death domain-adaptor protein system. The most likely interpretation of these observations is that the apoptotic response to TNF requires SAPK/JNK signaling in addition to that of adaptor proteins (see below). Consistent with this hypothesis are the preliminary studies that show that CD95-induced apoptosis is impaired in NPD cells and that this defect is reversed upon restoration of ASMase activity by retroviral transduction of the ASMase gene (Peña LA and Kolesnick R, unpublished observation).

LINKING THE SAPK/JNK PATHWAY, ICE/ced-3 PROTEASES, AND APOPTOSIS

The data described above indicate that transmembrane signaling initiated by cytokines and environmental stress involves the activation of the sphingomyelin pathway to effect apoptosis. However, recent studies also show that cytokine receptors respond to ligand by formation of multiprotein complexes at the receptor, which mediate the apoptotic response. In the case of CD95 and the TNF receptor, complex formation occurs by the binding of cytoplasmic proteins containing death domains to the death domain of these receptors (Fig. 2). In the case of the TNF receptor, a protein termed TRADD binds via its death domain to the receptor and to another adaptor protein termed FADD (also termed MORT1) [7, 8, 60]. CD95, however, does not require TRADD and binds FADD/MORT1 directly. In addition to its death domain, FADD/MORT1 contains a region termed a DED, another protein–protein interaction motif, which links to the DED of an ICE-like protease termed FLICE/MACH1. Ligand binding to the

TNF receptor and CD95 induces the formation of the death domain adaptor protein complex within seconds to minutes. Further, overexpression of these proteins individually initiates apoptosis and dominant-interfering mutations block ligand-induced apoptosis. Hence, it has been postulated that formation of the ligand-dependent multi-protein complex that links directly to an ICE protease is sufficient to initiate a protease cascade that effects apoptosis directly. This mechanism would be analogous to that of complement or clotting.

Other evidence, however, indicates that in some cellular systems the death domain adaptor protein mechanism may link to and utilize the sphingomyelin pathway for induction of apoptosis. As stated above, deletions and mutations of the death domain region of the TNF receptor [21] and CD95 [28], which abrogate induction of apoptosis, also block ASMase activation. Further, Dixit and co-workers reported that overexpression of dominant negative FADD/MORT1 blocks ligand-induced ceramide generation [8]. Treatment with ceramide analogs bypassed the effect of dominant-negative FADD/MORT1 and restored apoptosis. In at least one system, sphingomyelinase activation may, in fact, be downstream of an ICE protease, since REAPER-induced ceramide generation is blocked by the ICE inhibitor Z-VAD-fmk [61]. Taken together, these studies indicate that in some cell systems, ceramide generation downstream of the death domain adaptor protein system may participate as mediator of the apoptotic response. The notion that induction of apoptosis via cytokine receptors may involve ceramide generation is also supported by the observations that disruption of ceramide signaling through the SAPK/JNK cascade by dominant negative kinases or by transmodulation with SPP, as described above, blocks TNF-induced apoptosis without blocking TNF-induced ceramide generation [32, 55]. Hence, ceramide generation may be necessary for this mode of apoptosis, but how this function is coordinated with the ICE/ced-3 proteases activated directly through FLICE/MACH1 remains unknown (Fig. 2).

The generation of ceramide by environmental stresses, such as UV and ionizing radiation, may occur via a different mechanism. Ionizing and UV radiation have direct effects on the cell membrane [62, 63] and are capable of generating ceramide in isolated membranes ([29]; Mathias S and Kolesnick R, unpublished observations), which would imply that ceramide generation may be independent of a death domain adaptor protein system. In these instances, the sphingomyelin pathway may act in concert with as of yet unidentified accessory pathways that impact on ICE/ced-3 proteases.

The models of transmembrane signaling of apoptosis discussed here represent an effort to provide a paradigm that unifies the current state of the knowledge in this rapidly advancing field. Whereas a variety of mechanisms of transmembrane signaling seem to function coordinately in some cellular systems, there is a great deal that remains unknown, and only new information will settle some of the controversies and uncertainties that exist in the field. Since apoptosis plays a major role in the pathogenesis of disease and its management, it is important to advance the knowledge in this field. Improved understanding of various apoptotic signaling mechanisms and their coordinated function may yield opportunities for pharmacological interventions with important potentials for clinical application.

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