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Ceramide: A Stress Signal and Mediator of Growth Suppression and Apoptosis

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Abstract A novel pathway termed the sphingomyelin cycle has been identified whereby membrane sphingomyelin is hydrolyzed in response to multiple extracellular stimuli (such as tumor necrosis factor α) which cause activation of regulated sphingomyelinases. The product, ceramide, has emerged as a second messenger that mediates many of the cellular effects of these extracellular stimuli. An intriguing relation exists between activation of the sphingomyelin cycle and the action of multiple stress stimuli that induce growth arrest and programmed cell death. Exogenously administered ceramide mimics these growth-suppressing effects, including the induction of apoptosis. This review will highlight the role of the sphingomyelin cycle in signal transduction and will focus on the role and function of ceramide in the regulation of cell growth in general and apoptosis specifically. © 1995 Wiley-Liss, Inc.

Key words: sphingomyelin cycle, signal transduction, leukemia, membrane lipids, apoptosis

Membrane lipids are composed primarily of phospholipids, sphingolipids, and cholesterol. Historically the major role of lipids was thought to be structural, whereby they establish a barrier for cell permeability as well as a matrix for the association of membrane proteins. With the discovery of the phosphatidylinositol (PI) cycle, membrane phospholipids were thrust into a central role in signal transduction and cell regulation. It is now recognized that membrane phospholipids are hydrolyzed by one of several different phospholipases such as phospholipase C, phospholipase D, and phospholipase A_2 which generate a variety of products [Nishizuka, 1992; Berridge and Irvine, 1989; Rhee et al., 1989; Majerus et al., 1986; Dennis et al., 1991; Liscovitch, 1992]. These include diacylglycerol (DAG), inositol trisphosphate (IP_3), phosphatidic acid, platelet activating factor, and several eicosanoids which have been shown to function as important signal transducing molecules. When studies of protein kinase C (PKC) regulation led to the identification of sphingosine as an inhibitor of the enzyme [Hannun et al., 1986], it became critical to evaluate sphingolipids as signal transducing molecules. These studies led to the discovery of the sphingomyelin cycle, whereby membrane sphingomyelin is hydrolyzed and ceramide is generated in response to several extracellular agents [Hannun, 1994]. This provided an initial insight into a role for these molecules in signal transduction. Ongoing studies support a critical role for sphingomyelin hydrolysis as a stress-activated signaling mechanism with an important role for ceramide in growth suppression and apoptosis.

THE SPHINGOMYELIN CYCLE

The sphingomyelin cycle was initially described by Okazaki et al. [1989] in human HL-60 leukemia cells, whereby early and reversible hydrolysis of sphingomyelin was observed in response to vitamin D3. This was shown to occur through activation of a neutral sphingomyelinase and was accompanied by concomitant generation of ceramide [Okazaki et al., 1990]. The sphingomyelin cycle is completed by regeneration of sphingomyelin probably by the transfer of a choline head group back to ceramide from phosphatidylcholine. To date, several other inducers have been shown to be coupled to the sphingomyelin signal transduction cycle, including tumor necrosis factor α (TNFα), γ-interferon, dexamethasone, complement, interleukin-1, nerve growth factor, and brefeldin A [Kim

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et al., 1991; Ballou et al., 1992; Mathias et al., 1991; Quintans et al., 1994; Linardic et al., 1992]. The mechanism of this coupling is unknown, but in the case of TNFα the pathway is initiated by the action of TNFα on its 55 kilodalton receptor [Yanaga and Watson, 1992; Wiegmann et al., 1992], leading to phospholipase A_2 activation, generation of arachidonic acid, and subsequent activation of sphingomyelinase [Jayadev et al., 1994].

Since its discovery, the sphingomyelin cycle has been implicated in mediating the effects of several of these inducers leading to important biochemical and cellular effects. These include c-myc downregulation, NF-KB activation, upregulation of cyclooxygenase, and inhibition of phospholipase D [Wolff et al., 1994; Schütze et al., 1992; Betts et al., 1994; Dbaibo et al., 1993; Ballou et al., 1992; Venable et al., 1994; Nakamura et al., 1994; Gomez-Muñoz et al., 1994]. These (and other) biochemical occurrences lead to important biological end points such as growth arrest. This was evident in HL60 cell terminal differentiation [Okazaki et al., 1990], fibroblast senescence, and, most intriguing and the focus of this review, apoptosis [Obeid et al., 1993].

APOPTOSIS

Apoptosis (from the Greek term for falling of the leaves) has recently become recognized as an important physiologic mechanism for regulation of cell growth, development, oncogenesis, and immunodeficiency [for comprehensive reviews see Gerschenson and Rotello, 1992; Kerr and Harmon, 1991; McConkey and Orrenius, 1994; Wyllie et al., 1992]. Cells undergo apoptosis in response to a number of physiologic and pharmacologic extracellular stimuli. A process of orderly cell death occurs whereby chromatin becomes compact; then there is condensation of the cytoplasm, nuclear fragmentation, and the development of cellular protuberances that contain well preserved organelles. These structures separate and become apoptotic bodies which are then phagocytosed by adjacent cells.

Significant insight has developed into the regulation of apoptosis; however, most of our knowledge to date pertains to downstream occurrences in the program of cell death. These include the activation of a magnesium-dependent, zinc-inhibited endonuclease that digests DNA at internucleosomal locations [Kerr and Harmon, 1991]. Certain oncogenes such as c-fos and c-myc have been shown to induce apoptosis under conditions where cells receive a conflicting signal to growth arrest [Harrington et al., 1994]. Bcl-2, an oncogene whose translocation and deregulation has been implicated in human B-cell lymphomas, protects from cell death induced by most but not all inducers of apoptosis [Reed, 1994]. The mechanism of this protection is yet to be defined. The tumor suppressor protein p53 is a transcription factor that functions as a checkpoint at the G1 phase of the cell cycle in response to DNA damage. In some cell types p53 has been shown to be essential for apoptosis in response to DNA damage, whereas in other cell types it induces growth arrest [Vogelstein and Kinzler, 1992].

SIGNAL TRANSDUCTION IN APOPTOSIS

A significant body of data has recently accumulated which implicates the better characterized signaling pathways in modulating (enhancing or protecting from) programmed cell death (see Fig. 1 for a schematic illustration). These include 1) the ability of a sustained release or influx of calcium to activate an endonuclease and induce cell death in thymocytes and immune cells [Lynn et al., 1989]. This occurs in response to dexamethasone or calcium ionophores [for references, refer to McConkey et al., 1992]. This should be distinguished from the transient calcium increases that can occur early in signal transduction and concomitant with activation of protein kinase C (PKC) which may actually protect from apoptosis (next section). 2) The cAMP and protein kinase A pathway is clearly implicated in programmed cell death. In thymocytes and myeloid leukemia cells, agents that stimulate adenylate cyclase or cause elevation of cAMP cause apoptosis [McConkey et al., 1990; Lanotte et al., 1991]. Microinjection of the catalytic subunit of cAMP-dependent protein kinase has been shown to induce apoptosis [Vintermyr et al., 1993]. 3) The role of the DAG/PKC pathway in programmed cell death has been more intensely investigated, with several studies demonstrating a protective role for phorbol esters in programmed cell death. Phorbol esters block calcium ionophore- or glucocorticoid-induced DNA fragmentation in thymocytes [McConkey et al., 1989, 1992] and spontaneous apoptosis of chicken Bursa cells [Asakawa et al., 1993]. Phorbol esters also block cAMP-induced thymocyte programmed cell death [McConkey et al., 1990]. PKC has also been shown to have a role in inhibition of apoptosis by GM-CSF and IL-3 in megakaryoblastic leukemia cells [Rajotte et al., 1992]. All the above studies have in com-

mon the activation of PKC by phorbol esters. However, prolonged phorbol ester treatment with concomitant downregulation of PKC may enhance or cause programmed cell death [Ojeda et al., 1992] and should be distinguished from the above findings. 4) Studies suggest that activation of phospholipase A_2 may participate in mediating cell killing. This has been especially studied in the case of TNFα [Larrick and Wright, 1990], where inhibitors of phospholipase A_2 inhibit TNF-induced cytotoxicity [Palombella and Vilcek, 1989] and where TNFα fails to release arachidonic acid in cell lines resistant to the cytotoxicity of TNFα [Neale et al., 1988; Hayakawa et al., 1993]. 5) Protein phosphatases 1 and 2A have been implicated in programmed cell death. Inhibitors of these protein phosphatases such as okadaic acid and calyculin A were shown to prevent irradiation-induced DNA fragmentation in Burkitt's lymphoma cells [Song and Lavin, 1993]. Okadaic acid, however, has also been shown to induce apoptosis in hepatocytes [Boe et al., 1991]. 6) Several lines of evidence indicate that tyrosine kinase activity suppresses apoptosis. For example, temperature-sensitive v-abl transfectants of an IL-3–dependent murine mast cell line show that, upon IL-3 withdrawal at the restrictive temperature, cells undergo apoptosis. This is inhibited at the permissive temperature, indicating that abl inhibits programmed cell death [Evans et al., 1993]. The use of tyrosine kinase inhibitors has provided further evidence for a role for tyrosine kinases in mediating the protective effects of IL-2 and IL-3 on apoptosis, probably by regulating the expression of bcl-2 [Otani et al., 1993]. In summary, evidence is accumulating as to the role of several signal transduction pathways in programmed cell death (Fig. 1), indicating a possible requirement for more than one signal and the existence of more than one pathway modulating this complex cellular process.

CERAMIDE: A MISSING LINK IN APOPTOSIS?

As discussed above, an expanding wealth of information exists relating signal transduction pathways to apoptosis. However, little insight is available on the earliest and most direct signals that link extracellular stimuli to the downstream occurrences in apoptosis. Sphingomyelin hydrolysis and ceramide generation may provide such a link connecting stress stimuli to the intracellular machinery of apoptosis. Indeed, in initial studies on the cellular activities of cell-permeable ceramides, it was observed that in addition to their effects on cell differentiation, these compounds exerted potent antiproliferative activities accompanied by significant cytotoxicity [Okazaki et al., 1990]. Two observations prompted further investigation of this cytotoxicity and its mechanism. First, this cytotoxicity demonstrated significant structural specificity in that D-*erythro*-ceramide and related synthetic agonists were active, whereas closely related structural and stereoisomers such as dihydroceramide were inactive [Bielawska et al., 1993]. Second, this cytotoxicity mimicked closely the cytotoxicity of TNFα in that it occurred in the same susceptible cell lines and under *similar* conditions.

Thus, initial studies showed that TNFα induced apoptosis in the same leukemia cells where TNFα induced sphingomyelin hydrolysis and ceramide generation. Exogenously administered cell-permeable ceramide was able to induce apoptosis as measured by nuclear fragmentation and thymidine release, thus mimicking the effects of TNFα. These studies established the ability of ceramide to induce apoptosis and suggested a role for ceramide in mediating apoptotic activities of TNFα and other agents [Obeid et al., 1993].

Indeed, the sphingomyelin cycle has been implicated in apoptosis in many cell systems [Jarvis et al., 1994] and in response to additional inducers such as radiation, dexamethasone, antibody cross-linking and in response to serum withdrawal [Quintans et al., 1994; Jayadev et al., in press]. Other evidence points to a role for ceramide in HIV-induced T-cell apoptosis. Ceramide levels increase in CEM cells upon HIV infection [Van Veldhoven et al., 1992], and ceramide induces apoptosis in this cell line [Obeid et al., 1993].

Studies on the effects of serum withdrawal on the sphingomyelin/ceramide pathway have been quite informative. In these studies [Jayadev et al., in press], serum deprivation of serum-dependent cell lines (such as Molt-4 leukemia cells) results in cell cycle arrest and apoptosis which are accompanied by dramatic and prolonged increases in the intracellular levels of ceramide (reaching fifteenfold over baseline levels) due to activation of a membrane sphingomyelinase and to significant hydrolysis of membrane sphingomyelin. The addition of exogenous ceramides to these cell lines resulted in significant apoptosis as well as significant cell cycle arrest. Further studies demonstrated that this

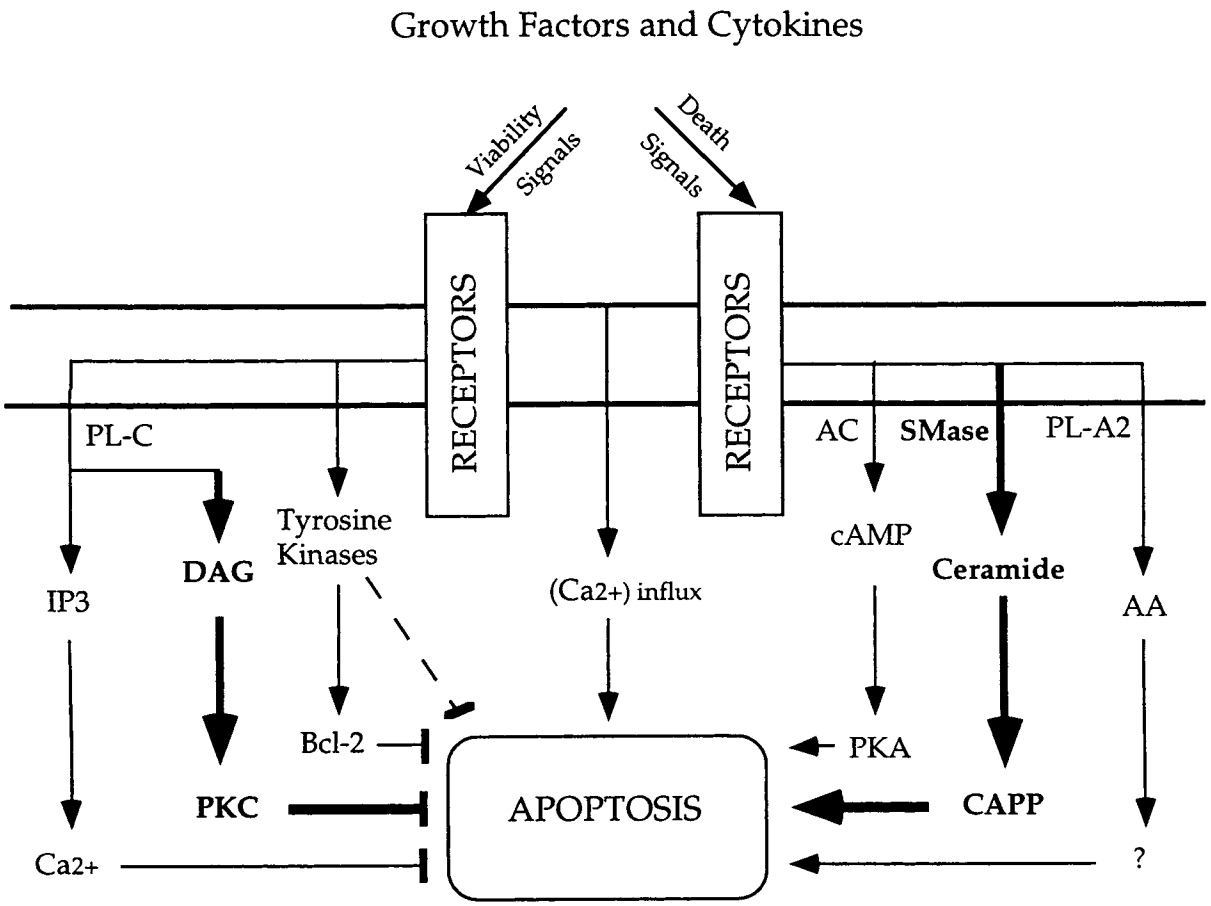

Fig. 1. Schematic illustration of proposed roles for various signal transduction pathways in the regulation of apoptosis. Activation of the phospholipase C (PL-C)/PKC pathway and activation of receptor and nonreceptor tyrosine kinases appears to predominantly improve viability of cells and, in many cases, protect from the induction of apoptosis. On the other hand, activation of the sphingomyelinase/ceramide pathway and the phospholipase A2/arachidonate pathway appears to be involved predominantly in induction of cytotoxicity. Apoptosis is also modulated by activation of the cyclic AMP/protein kinase A pathway and by calcium signaling. These effects with representative references are discussed in the text. In this simplified scheme, interactions and cross-talk between different pathways are not shown. Also, the predominant effect of each pathway is represented, although it is possible that different pathways, under different conditions, may modulate apoptosis in different directions.

G_0/G_1 cell cycle arrest was accompanied by significant dephosphorylation of the retinoblastoma gene product (Rb), which has been implicated as an important checkpoint regulator of cell cycle progression. Additional studies [Dbaibo et al., in press] have demonstrated that the cell cycle arrest in response to ceramide is probably mediated through the dephosphorylation of Rb since cell lines that lack Rb or cell lines in which Rb had been rendered nonfunctional through the action of Rb-binding proteins became resistant to the cell cycle arrest induced by ceramide. However, this pathway was independent of ceramide-induced apoptosis. Moreover, diacylglycerols and phorbol esters prevented ceramide-induced apoptosis but not ceramide-induced cell cycle arrest (see Fig. 2). Therefore, these studies are beginning to distinguish at least two distinct outcomes of ceramide action which appear to involve distinct intracellular pathways and mechanisms.

In additional studies, it was shown that apoptosis induced by TNFα and ceramide in multiple cell lines was reversed by phorbol ester or diacylglycerol treatment [Obeid et al., 1993]. These studies suggested that the diacylglycerol/PKC pathway opposes the effects of ceramide on apoptosis. This latter observation has generated significant insight into ceramide-induced apoptosis. First, it is now clear that the effects of ceramide are not due to nonspecific lipid interactions since diacylglycerol (another lipid) pre-

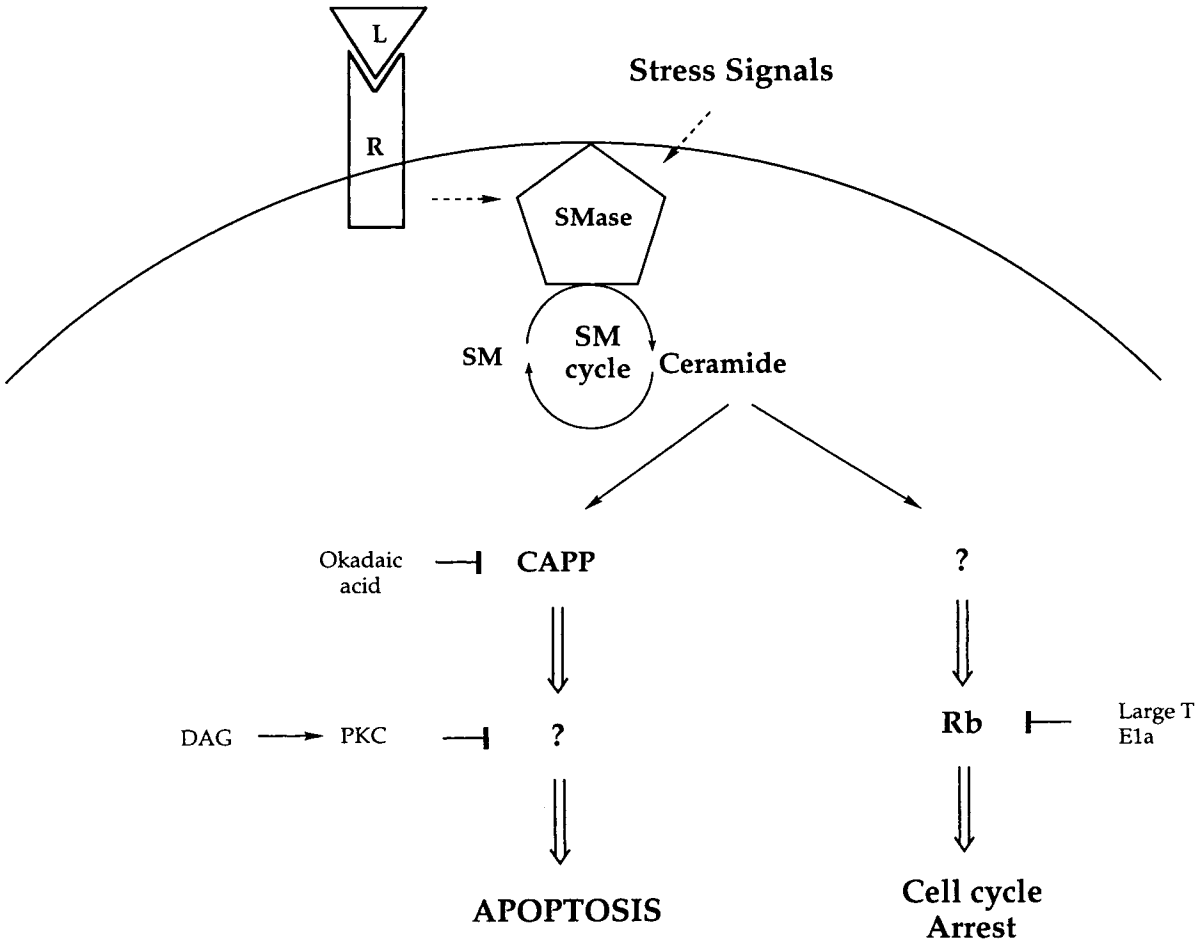

Fig. 2. Proposed role for the sphingomyelinase/ceramide pathway as a stress signal transduction system. Emerging data suggest that various growth suppressor cytokines as well as cytotoxic agents and molecules activate the sphingomyelin cycle and induce ceramide accumulation. As discussed in the text, ceramide induces either cell cycle arrest through an Rb-dependent pathway or apoptosis through a distinct mechanism. The effects of ceramide on cell cycle arrest can be abrogated by inhibition/sequestration of Rb (e.g., by binding Rb to viral proteins such as T and E1A), whereas apoptosis can be selectively inhibited by activation of the protein kinase C pathway. These results suggest that ceramide may function as a sensor of stress and stress-inducing agents. The specific biologic outcomes (such as cell cycle arrest, apoptosis, or cell senescence) may depend on which downstream pathways are activated as well the role of other signaling pathways in modulating responsiveness to ceramide.

vents the effects of ceramide. Moreover, the effects of ceramide on apoptosis are specific to D-*erythro*-ceramide in that the closely related D-*erythro*-dihydroceramide (which lacks the trans 4-5 double bond of ceramide) is inactive. Thus, the ability of ceramide to induce apoptosis is due to specific structural requirements and not to nonspecific hydrophobic interactions. Second, the ability of diacylglycerol to oppose the effects of ceramide demonstrates that apoptosis is not a necessary outcome of ceramide action but that it is a regulated outcome of diverse signal transduction pathways. Third, the outcome of apoptosis may be more responsive to the relative concentrations of ceramide and diacylglycerol (and possibly other intracellular mediators) rather than the absolute levels of either ceramide or diacylglycerol. Finally, these studies raise the possibility that the ceramide and diacylglycerol pathways may converge on a unique downstream target which may function as a switch in the regulation of apoptosis and cell viability (Fig. 2).

MECHANISM OF CERAMIDE ACTION

The mechanism by which ceramide induces apoptosis remains to be elucidated. Insight is forthcoming from a number of studies that ad-

dress targets of ceramide action. Ceramide has been shown to activate a cytosolic serine/threonine protein phosphatase termed ceramide-activated protein phosphatase (CAPP) [Dobrowsky and Hannun, 1992]. CAPP has been purified partially from brain and tissue culture cell lines and has been shown to share several properties with protein phosphatase 2A [Dobrowsky et al., 1993]. Thus, CAPP is inhibited potently by okadaic acid and related inhibitors of PP2A, cochromatographs with PP2A, and is not sensitive to the action of inhibitor 1 (an inhibitor of PP1 but not PP2). Indeed, purified PP2A is very sensitive to activation by ceramide but not by related sphingolipids. Importantly, ceramide was shown to activate in vitro trimeric PP2A (composed of A, B, and C subunits) but not monomeric PP2A (which constitutes the catalytic subunit) or dimeric PP2A (the catalytic C subunit in association with an A subunit of unknown function). Thus, the presence of the B regulatory subunit is essential for imparting ceramide responsiveness to PP2A [Dobrowsky and Hannun, 1993].

Further studies are beginning to implicate CAPP in at least some of the cellular activities of ceramide [Wolff et al., 1994]. For example, the ability of ceramide to induce apoptosis and to downregulate the c-myc protooncogene are inhibited by low concentrations of okadaic acid and are accompanied by cellular activation of PP2A. In addition, the structural specificity of activation of CAPP in vitro parallels closely the structural specificity of induction of apoptosis and c-myc downregulation. Thus, D-*erythro*-cermiade activates CAPP in vitro, induces c-myc downregulation, and causes apoptosis, whereas D-*erythro*-dihydroceramide is inactive in all these assays. These studies point strongly to a role for CAPP in the regulation of apoptosis and c-myc.

In addition to CAPP, ceramide has been shown to activate PKCζ in vitro [Lozano et al., 1994], although the extent and specificity of this activation has not been determined. Interestingly, a role for ceramide-induced activation of PKCζ has been proposed in the regulation of NF-KB [Diaz-Meco et al., 1993]. Also, ceramide has been shown to activate a membrane proline-directed protein kinase in intact cell systems and in cell free systems [Mathias et al., 1991]. However, the partially purified enzyme does not appear to respond to ceramide [Liu et al., 1994]. Moreover, the structural specificity of activation of this enzyme by ceramide has not been examined. This kinase may play a role in regulation of EGF receptor phosphorylation in response to ceramide and TNFα [Mathias et al., 1991]. These studies are beginning to provide initial insight into possible candidates for immediate targets for the action of ceramide.

CONCLUSIONS

These recent studies on the role and function of ceramide as a novel second messenger are beginning to delineate a novel intracellular pathway of cell regulation which is activated in response to a variety of extracellular stimuli and agents. We can already discern important biologic roles for this pathway especially in the regulation of growth suppression and apoptosis.

An examination of the spectrum of inducers of sphingomyelin hydrolysis and ceramide generation discloses a variety of growth suppressor cytokines such as TNFα and γ-interferon agents with potent apoptotic activities such as dexamethasone [Quintans et al., 1994] and TNFα, cytotoxic pharmacologic agents such as Ara-C [Strum et al., 1994], radiation [Quintans et al., 1994; Haimovitz-Friedman et al., 1994], and growth factor and serum factor withdrawal. Therefore, sphingomyelin hydrolysis and ceramide generation may represent a common downstream sensor and/or effector mechanism for the action of this diverse group of extracellular agents and insults. The exact mechanisms involved in regulation of sphingomyelin hydrolysis in response to this group of inducers are not well defined and may actually represent diverse and distinct mechanisms converging on sphingomyelin hydrolysis.

In this context, it appears that ceramide may not be a specific inducer of apoptosis but a more generalized inducer of growth suppression. That is, ceramide may function more as a responder to these various stress agents, and the cellular outcome of this stress pathway may depend not only on ceramide generation but also on the participation of other signal transduction mechanisms. This has been worked out best in the case of diacylglycerol, which appears to overcome ceramide-induced apoptosis but not ceramide-induced cell cycle arrest. Therefore, the combined generation of ceramide and diacylglycerol steers the cells away from apoptosis and towards cell cycle arrest. This may allow cells to recover from insult/injury and resume normal growth under more favorable conditions. Delineation of such a stress-response pathway is supported by recent studies which indicate that jun kinase (JNK) may function as a downstream target for

sphingomyelinase [Kyriakis et al., 1994]. JNK has also been termed SAPK (for stress-activated protein kinase) in recognition of its activation and response to a number of stress-inducing agents. This list of activators of SAPK bears a close resemblance to the list of activators of sphingomyelin hydrolysis and ceramide generation [Kyriakis et al., 1994].

The current challenge is to dissect mechanisms involved in sphingomyelin hydrolysis and ceramide generation. Such studies should provide biochemical and molecular insight into this novel pathway of cell regulation. In addition, identification of additional components of this pathway should provide important insight into the regulation of growth suppression and apoptosis. Such pathways may prove critical to the study of growth suppression as various mitogenic pathways have contributed to the understanding of cell growth and proliferation.

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