Ceramide and the eukaryotic stress response

Y. A. Hannun* and L. M. Obeid†

*Departments of Medicine and Cell Biology, Duke University, Durham NC 27710, U.S.A., and †Durham Veteran Affairs Geriatric Research and Clinical Centre, Durham, NC 27710, U.S.A.

Introduction

Sphingolipids constitute a major class of eukaryotic membrane lipids with a great diversity in structure that probably portends important functional consequences. The defining moiety of sphingolipids is a sphingoid base (long-chain amino base), such as sphingosine in mammalian tissues or phytosphingosine in yeast and plants. Acylation of the sphingoid base results in the formation of ceramides, which in turn serve as precursors to complex species of phosphosphingolipids and glycosphingolipids. Many studies over the last several decades have defined the structural composition of over 300 distinct species of sphingolipids, which are defined by the unique substituents at the 1-hydroxyl position of the molecule. In addition, multiple lines of investigation have correlated the expression of specific sphingolipids with various developmental and differentiation stages as well as with specific biological responses, such as cell contact response, cell recognition, receptor interactions, differentiation and cell transformation.

In addition to these functions of sphingolipids, evidence over the last decade has begun to point to important roles for sphingolipids in signal transduction and cell regulation [1-5].

Abbreviations used: NGF, nerve growth factor; NF, nuclear factor; PI, phosphatidylinositol; PP, protein phosphatase; TNF, tumour necrosis factor.

Received 2 July 1997
ceramide formation from sphingomyelinases, which appears to play important roles in the regulation of cell death, cell differentiation and cell senescence. This review will highlight key features of this pathway, supporting evidence for the regulation and function of this pathway, and important questions that arise from this formulation.

Inducers of ceramide formation: activation by diverse stress stimuli

Initial studies aimed at discovering and defining regulated metabolism of sphingolipids led to the observation that the action of 1,25-dihydroxy vitamin D₃ on human leukaemia cells (HL60) resulted in relatively early hydrolysis of membrane sphingomyelin and reversible accumulation of ceramide occurring over a period of 1–3 h in what has now been termed the sphingomyelin cycle. Subsequent studies demonstrated that tumour necrosis factor α (TNF α) and interferon γ also induce sphingomyelin hydrolysis and ceramide formation, albeit with faster kinetics in HL60 cells. In this cell line, it appeared that selective inducers of monocytic differentiation shared in the ability to induce sphingomyelin hydrolysis and ceramide accumulation, and this was not observed with inducers of other differentiation pathways, such as phorbol esters, which cause macrophage-like differentiation, or retinoic acid, which causes neutrophilic differentiation.

Over the last few years, the list of inducers of ceramide formation and/or sphingomyelin hydrolysis has expanded rapidly. These include activation of the apoptotic Fas receptor, interleukin-1, dexamethasone, IgM-directed antibodies, heat, ionizing irradiation, UV radiation, photodynamic therapy, heat stress, several cytotoxic agents (such as brefeldin A, cytosine arabinoside, vincristine and daunorubicin) and nerve growth factor (NGF). These effects on sphingomyelin and ceramide metabolism have been observed in several cell lines, including normal human diploid fibroblasts, lymphocytes, leukaemia cells, cancer cells, and neuronal tissues and cell types [2].

The common theme uniting these inducers involves either stress stimuli or agents of injury and toxicity. Therefore, the formation of ceramide appears to be a common occurrence in a variety of situations in which cell function is stressed or perturbed. In most of the model cell systems in which ceramide formation has been observed, the inducers of ceramide formation also appear to cause either terminal cell differentiation, cell-cycle arrest, or apoptosis.

Kinetics of ceramide formation

The kinetics of ceramide accumulation appear to vary depending on cell type and inducer. However, two distinct phases of ceramide formation have been observed. With TNF α, NGF and interleukin-1, reversible hydrolysis of sphingomyelin and accumulation of ceramide have been observed to occur over a period of 5–60 min. This ceramide appears to form from the hydrolysis of sphingomyelin, and it appears to be re-incorporated into sphingomyelin, thus completing the sphingomyelin cycle. These studies have not ruled out the involvement of ceramide in other metabolic pathways of sphingolipid metabolism (such as a portion of the ceramide being hydrolysed further to yield sphingosine and subsequently sphingosine 1-phosphate or ceramide being phosphorylated to yield ceramide 1-phosphate). The physiological function of this reversible phase of ceramide formation has not been clearly determined, but it appears to be closely related to the long-term action of ceramide inducers on cell growth, viability and differentiation.

A second and more protracted phase of ceramide formation has been described in several cell systems in response to TNF α, serum deprivation, cytotoxic chemotherapy, Fas receptor activation, dexamethasone and NGF. This phase is characterized by progressive accumulation of ceramide over several hours with no discernible reversibility [2]. Ceramide attains levels of approx. 3–15-fold over baseline. This accumulation has been closely correlated with the induction of cell-cycle arrest, cell senescence or apoptosis. The specific mechanisms involved in this phase of ceramide formation have not been clearly defined, but they appear to involve the activation of at least membrane neutral sphingomyelinase and possibly the regulation of other enzymes of ceramide metabolism.

Regulation of magnesium-dependent neutral sphingomyelinase

Several sphingomyelinases have been described. The best studied is the acid sphingomyelinase, which functions optimally at a pH of 4–5 and appears to reside primarily but not exclusively in the lysosomes. Deficiency of this enzyme is the cause of the inherited form of sphingolipidoses (Neimann–Pick disease). Some studies have sug-
gested activation of this enzyme in response to stress stimuli such as ionizing radiation and activation of the Fas receptor. The acid sphingomyelinase has been purified and cloned, and transgenic knock-outs in the enzyme have been established in mice. Mice deficient in this enzyme show some resistance to apoptosis of endothelial cells in response to high doses of ionizing radiation. The mechanisms involved in regulating acid sphingomyelinase remain poorly defined.

A membrane-associated neutral sphingomyelinase has been known for several years, and it has been shown to have a wide tissue distribution and to depend on magnesium for optimal activity. Several studies have suggested activation of this enzyme in response to TNF α, activation of the Fas receptor, serum deprivation, cytotoxic chemotherapy, dexamethasone, antibodies to IgM and other inducers of ceramide. The neutral sphingomyelinase has not been studied extensively. Our recent data show that the partially purified enzyme is dependent on anionic phospholipid for optimal activation as well as bivalent cations, especially magnesium.

In a study of regulation of this enzyme, it was noted that the tripeptide glutathione causes complete inhibition of this enzyme at approx. 2–4 mM. The concentration dependence of this inhibition is very steep, such that 1 mM glutathione shows no detectable inhibition whereas 4 mM shows total inhibition. This inhibition was specific to glutathione and neutral sphingomyelinase such that other reducing agents did not inhibit the neutral sphingomyelinase, and reciprocally glutathione did not inhibit the acid sphingomyelinase. Structure-function studies demonstrate that the inhibition does not require the terminal glycine of glutathione and is mediated by the γ-glutamylcysteine and possibly by cysteine alone. Since the resting cellular levels of glutathione in normal cells have been estimated at 3–20 mM, the inhibition of neutral sphingomyelinase by glutathione suggests that in normal resting cells the enzyme exists in an inhibited form as long as glutathione levels are maintained. Also, these results suggest that once glutathione levels drop to below 2 mM, the enzyme experiences acute activation due to relief of inhibition by glutathione. This was examined by inducing cellular depletion of glutathione through the use of buthionine sulfoximine, which inhibits the rate-limiting step in glutathione formation, the action of γ-glutamylcysteine synthase. Treatment of Molt-4 leukemia cells with buthionine sulphoximine resulted in time-dependent depletion of glutathione. This was accompanied by similar kinetics of sphingomyelin hydrolysis and accumulation of ceramide, suggesting activation of neutral sphingomyelinase. Taken together, these results suggest a close link between cellular levels of glutathione and the status of neutral sphingomyelinase. They also suggest an intimate coupling between oxidative stress and sphingolipid signalling through the ceramide pathway. According to this hypothesis, oxidative stresses that result in depletion of glutathione will cause activation of neutral sphingomyelinase. Reciprocally, many activators of neutral sphingomyelinase may achieve this through depletion of cellular glutathione. The ramifications of these hypotheses are currently being tested.

Biological functions of ceramide
As a sphingolipid-derived product and as a putative lipid effector molecule, ceramide has been examined for its effects on diverse cellular functions. These studies have been accomplished by either treatment of cells with short-chain, cell-permeable analogues of ceramide or by modulating endogenous ceramide levels through the regulation of various enzymes of ceramide metabolism. These different manoeuvres have shown that elevations in ceramide levels are capable of causing a G₁/G₂ cell-cycle arrest, which appears to be mediated through induction of dephosphorylation of the retinoblastoma gene product. In a study examining the effects of ceramide on cell viability and toxicity, it was shown that ceramide causes all the hallmarks of apoptotic cell death, as demonstrated by morphological features, DNA fragmentation and exteriorization of phosphatidylserine. Increases in ceramide levels have been shown to activate caspases (cell-death proteases), which mediate the effects of ceramide on apoptosis. In HL60 cells, the addition of ceramide has been shown to cause monocytic differentiation, especially in the presence of low concentrations of vitamin D₃, whereas in neuronal cells, ceramide has been shown to regulate neuronal differentiation [2].

In all these studies, it should be noted that the effects of exogenous ceramides are consistent with a role for endogenous ceramide in mediating or at least regulating these events. This is supported by the following. (1) The specificity of
Mechanisms of ceramide action
Several in vitro targets for the action of ceramide have been suggested. These include (1) protein kinase Cζ, which has been related to the action of ceramide on nuclear factor (NF)-κB. This is a controversial area, with some studies showing that ceramide is capable of activating NF-κB and other studies dissociating ceramide from NF-κB. (2) Ceramide-activated protein kinase [5]. Recent studies have identified this kinase as kinase suppressor of Ras (KSR), and have suggested that this pathway may be important for regulating Rab kinase resulting in activation of mitogen-activated protein kinase. The extent of activation of mitogen-activated protein kinase by ceramide and by inducers of ceramide formation is, however, usually of a small magnitude, and its contribution to the overall biology in response to ceramide is undetermined.

Other studies have demonstrated the existence of a ceramide-activated protein phosphatase (PP) that appears to belong to the PP2A family of serine/threonine phosphatases [1]. Studies in human cells suggest that ceramide, but not dihydroceramide, can activate this phosphatase both in vitro and in cells. This enzyme is inhibited potently by okadaic acid, which has been shown to inhibit some of the effects of ceramide, especially on apoptotic cell death. In Saccharomyces cerevisiae, ceramide has been shown to activate a PP with similar biochemical properties and to cause cell-cycle arrest and growth suppression. The composition of this ceramide-activated PP in yeast has been determined through genetic studies and identified as Sit 4 for the catalytic subunit, TPD3 for the A-regulatory subunit, and CDC55 for the B-regulatory subunits. Ongoing studies are aimed at defining the cellular regulation of ceramide-activated PP in yeast and in mammalian cells and in defining its chemical regulation and mechanism of regulation in vitro by ceramide.

Conclusions
These and many other studies are beginning to define important pathways of sphingolipid metabolism that result in the regulation of sphingolipid-derived products. In the case of ceramide, examination of inducers of ceramide, the kinetics of formation of ceramide, mechanisms of regulation of ceramide formation, and the action of ceramide on cells leads to a general hypothesis implicating ceramide formation in various stress-activated pathways. According to this hypothesis, the action of many stress stimuli and/or cytotoxic agents results in accumulation of ceramide, possibly through various metabolic pathways. This accumulation then partakes in the response of cells to these agents. These responses include cell-cycle arrest, cell death, differentiation or cell senescence. It appears that the final response may be determined by other regulatory mechanisms, such as protein kinase C, Rb and Bcl-2. Current investigation is aimed at defining the biochemical and molecular mechanisms involved in regulating enzymes of ceramide formation in response to this diverse group of stress stimuli. The study of these enzymes suggest that individual enzymes may serve as distinct switches allowing the connection of a specific subset of stress stimuli to ceramide formation. Additional
studies are aimed at defining the mechanisms by which ceramide induces the distinct cell responses of cell-growth suppression, cell-cycle arrest, cell senescence and apoptosis. Hopefully, the study of these mechanisms will clarify the operation of this pathway and its significance in cell regulation and cell biology.

This work was supported in part by NIH Grant GM43825 and DOD Grant AIBS-516.


Received 10 July 1997