Chapter 11

CERAMIDE SIGNALING UNDER OXIDATIVE STRESS

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11.1 Introduction

Aerobic cells are constantly exposed to reactive oxygen and nitrogen species (ROS/RNS) that are generated under both physiologic and pathologic conditions. ROS include species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH). Under basal conditions, human cells produce about 2 billion O_2^- and H_2O_2 molecules per cell per day (1). ROS were once considered as only waste byproducts of aerobic metabolism or molecules of defense produced by host inflammatory cells against invading organisms. Now ROS are recognized as controlling key steps in cellular signal transduction cascades.[2,3] Therefore, ROS are involved in diverse biologic processes that include embryogenesis, normal tissue homeostasis, aging, and many human diseases. For example, ROS have been implicated as mediators of lung injury in Acute Respiratory Distress Syndrome (ARDS) (alveolar damage), asthma (airway epithelial damage), chronic obstructive pulmonary disease (COPD), and interstitial pulmonary fibrosis.[4-8] The role of ROS in illness was previously explained by the chemistry in which critical cell proteins and lipids were randomly oxidized and rendered metabolically inactive.[9] However, several groups, including ours, have recently demonstrated that ROS function as signaling molecules. As one example of the signaling properties of ROS in the airway, treatment of airway epithelial cells with exogenous H_2O_2, the agent commonly produced during lung inflammatory processes, has been shown to

H. J. Forman, J. Fukuto, and M. Torres (eds.), Signal Transduction by Reactive Oxygen and Nitrogen Species: Pathways and Chemical Principles, ©2003, *Kluwer Academic Publishers. Printed in the Netherlands*

activate EGF receptor tyrosine kinase but not the receptor's trafficking;[10,11] this presents a mechanism by which ROS directly mediate transduction of mitogenic signals to the nucleus. Our laboratory has also examined the role of ROS in another key physiologic process, apoptosis.[12-14] We found that H_2O_2, at physiological concentrations [50-250 nmoles/mg protein],[15,16] induces epithelial apoptosis via the ceramide signal transduction pathway. This work provides a direct link between two important aspects of mammalian stress responses: the generation of ROS and activation of the sphingomyelin/ ceramide cycle leading to apoptosis.[12-14] Indeed, a role for ceramide as a transducer of signaling by oxidative stress, and vice versa, has been demonstrated by other laboratories in different cell types.[17-19] Clearly, the interaction between oxidative stress and the ceramide pathway is taking a prominent role in biological processes and diseases where ROS are found. The objective of this chapter is to summarize the work that established the coupling of oxidative stress and ceramide production and illustrate where this coupling plays a role in disease. This chapter will emphasize important developments, elucidate basic principles and address key questions for future studies.

11.2 Reactive Oxidants Induce Apoptosis

Excessive accumulation of reactive oxidants is toxic, and the intracellular level of reactive oxidants is therefore tightly regulated by several antioxidants. Although antioxidant defenses are constitutively expressed in mammalian cells,[20] additional responses are mounted when the amount of environmental oxidants exceeds a threshold level, thereby becoming a threat to overall tissue integrity. Apoptosis may be one such cellular adaptive response.

Conflicting data related to oxidative stress and apoptosis may be the result of missing information regarding the various targets for reactive oxygen species as well as lack of data related to the different chemical modifications of these targets. Moreover, the targets may be differently expressed in various cells, thus leading to different end-results in terms of cell apoptosis. Recently, there has been a growing consensus that ROS as well as nitric oxide and its congeners play a key role in apoptosis. However, the precise nature of this role is unclear.

The generation of oxidative stress has been proposed as a critical event for death-inducing agents in the process of initiating their apoptotic activity. Specifically, depletion of glutathione (GSH), the most abundant intracellular thiol-containing small peptide, has been suggested to precede the onset of apoptosis induced by various agents. Moreover, it has been also suggested that depletion of GSH could be an early event in the commitment to apoptosis.[21-24] However, how depletion of GSH transmits apoptotic signals is yet unknown.

One of the most reproducible inducers of apoptosis is mild oxidative stress produced by H_2O_2, which is a ubiquitous molecule, freely miscible and able to cross cell membranes readily. It is present in several air pollutants, including the vapor phase of cigarette smoke. It is detected in the exhaled air of humans[25] and amounts of exhaled H_2O_2 are greater in subjects with lung inflammation[26-28] or who smoke cigarettes[29]. Importantly, several agonists increase H_2O_2 generation by epithelial cells, including cytokines (TNFα, IL1, and Fas ligand), cytotoxic agents, ionizing radiation and infections (e.g., HIV or bacteria). With the demonstration that apoptosis can be triggered by ceramide generation as a result of H_2O_2 interaction

with the cell membrane of lung airway epithelial cells,[12] the hypothesis that ceramide is a "coordinator" of eukaryotic stress responses[30] is strengthened. However, the mechanism by which ceramide induces apoptosis is still unknown.

11.3 Ceramide Metabolism

Initially, sphingolipids were thought to play predominantly a structural role as components of lipid bilayers. It is now apparent that sphingolipid metabolites, especially ceramide, sphingosine (SP), and sphingosine-1-phosphate (S1P), play essential roles in cell growth, survival and death.[31,32] Ceramide is a central molecule in sphingolipid metabolism (Figure 1). Sphingolipid biosynthesis begins in the endoplasmic reticulum (ER) with the condensation of serine and palmitoyl CoA, followed by reduction to dihydrosphingosine. Then, the N-acylation of dihydrosphingosine by ceramide synthase produces dihydroceramide that is then desaturated to generate ceramide.[33,34]

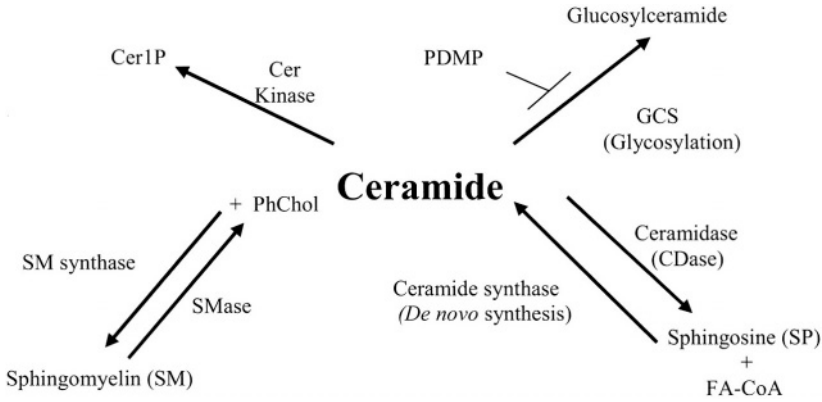

Figure 1: Metabolic pathways of ceramide.

Ceramide then serves as the precursor for all major sphingolipids in eukaryotes, such as sphingomyelin (SM), via SM-synthase, or glucosylceramide, via glucosyl ceramide synthase (GCS).[35]

The breakdown of complex sphingolipids results in the formation of ceramide through the action of sphingomyelinases (SMases). The catabolism of ceramide advances via the action of ceramidases (CDases), whereas the product sphingoid bases act as substrates for sphingosine kinases to form S1P or are recycled into ceramide and complex sphingolipids via the action of ceramide synthases[36,37] (see Figure 1).

Ceramide and S1P appear to mediate different cellular functions. Ceramide has been implicated in differentiation, cell cycle arrest, cellular senescence and apoptosis.[32,38] In contrast, its metabolic products sphingosine and particularly S1P have been implicated in cell proliferation,[39,40] protection from apoptosis,[41] induction of mitogenesis[42] and angiogenesis[43] (Figure 2). Thus, a key part of sphingolipid

metabolism is the balance between the relative and absolute cellular levels of the sphingolipid metabolites; the modulation of this balance by oxidants, cytokines and other factors has important life or death consequences for the cell.[44-46]

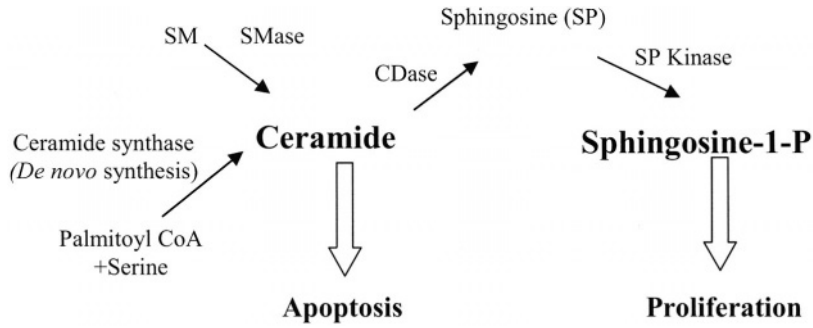

Figure 2: Biological effects of sphingolipid metabolites, ceramide and spingosine-1-phosphate (SIP).

11.4 Ceramide accumulation and apoptosis

Significant evidence suggests an important role for ceramide in mediating the apoptotic responses of several diverse agents, including oxidative stress. Importantly, the elevation in endogenous ceramide levels in response to these agents occurs prior to the onset of the execution phase of apoptosis when effector caspases are activated.[30,47,48] Furthermore, the addition of cell-permeable analogs of ceramide causes apoptosis in several cell lines.[49-52] The effects of ceramide are very specific such that dihydroceramide, which is the endogenous metabolic precursor to ceramide that lacks the 4-5 trans double bond, and has an uptake and metabolism similar to ceramide, does not cause apoptosis.[49,52-59] A role for ceramide in apoptosis is further supported by the demonstration that overexpression of glucosylceramide synthase (GCS) (Figure 1), which catabolizes ceramide by generating glucosylceramide, attenuated ceramide formation in response to TNF-α and several chemotherapeutic agents and protected the cells from apoptosis.[60,61]

Typically, ceramide accumulation results from the activation of one or more of the cellular SMases (Figure 3). Several of these sphingomyelin [SM]-specific phospholipase C (PLC) activities exist in mammalian tissues[52,55,62-65] and are distinguished according to their pH optimum and subcellular localization. At least eight SMases have been described. The best known includes the acidic SMase (aSMase), which displays a pH optimum of 4.5 and is confined to the lysosomes. Deficiency of this enzyme due to defects in the aSMase gene is responsible for the lysosomal disorder, Niemann-Pick disease,[66] which results in a massive accumulation of SM in the lysosomes and death in early childhood.[67] There are also various neutral SMases (nSMases) that act at neutral pH, are stimulated by Mg^{2+} or Mn^{2+} and are located in the plasma membrane, cytosol, endoplasmic reticulum or nuclear membranes.[68,69] Recently, an alkaline SMase in intestinal cells was also described.[70] Therefore, these isoenzymes differ not only in their catalytic properties

and subcellular localization, but probably also in their modes of regulation. SM hydrolysis by these SMases, triggered by the binding of extracellular ligands to cell-surface receptors or by agents that induce cellular stress, was believed to be the major source of elevated cellular ceramide levels.[71] Indeed, the Mg^{2+}-dependent nSMase that resides in the plasma membrane was thought to generate most of the ceramide used as a second messenger for apoptosis.[18,72-74]

However, recent studies indicate that other mechanisms of ceramide accumulation can occur that lead to apoptosis, beside activation of the plasma membrane nSMase. In fact, all of the metabolic pathways involved in ceramide generation, breakdown and incorporation into more complex lipids (Figure 1) may be regulated and play distinct roles in apoptosis.[46,75-79] For example, stress response-induced ceramide accumulation can occur due to activation of the de-novo pathway, catalyzed by ceramide synthase,[80] or sometimes as a result of inhibition of ceramide clearance through SM-synthase or ceramidases (CDases).[81-83] Fumonisin B1, an inhibitor of ceramide synthase and consequently of the de novo pathway, was able to block ceramide generation and apoptosis in response to extracellular agents such as retinoic acid, etoposide, angiotensin II, or daunorubicin.[84-89] It was also shown that cells treated with B13, a ceramidase inhibitor, responded with elevated ceramide levels and activation of the apoptotic cascade.[82,90] However, it is still unknown whether these metabolic pathways, beside nSMase, are also involved in oxidant-induced ceramide accumulation.

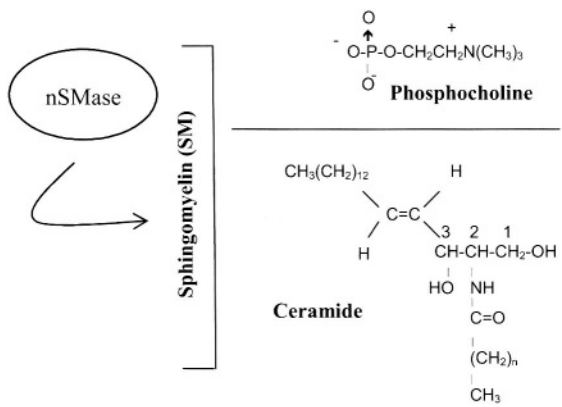

Figure 3: Hydrolysis of Sphingomyelin to ceramide.

11.5 Ceramide formation and reactive oxidants

Few reports have explored the role of ceramide in oxidant injury. Most studies that have used the apoptotic model of cell injury showed that increased ceramide generation due to diverse stimuli is mediated by activation of Smases.[30,91,92] Exposure of leukemic or endothelial cells to H_2O_2 resulted in increased ceramide generation with a concomitant decrease in SM content, which is suggestive of SMase activation.[93] Also, in an in vitro model of hypoxic injury to PC 12 cells,

reactive oxygen metabolites triggered ceramide generation via activation of nSMase.[91] In our laboratory, direct examination of nSMase activation during oxidative stress showed that H_2O_2 exposure concurrently induces both nSMase activity and elevated ceramide levels thereby leading to apoptosis in lung cells.[12,13]

However, Shah and co-workers[94,95] showed that hypoxia increases ceramide generation through ceramide synthase activation in LLC-PK1 cells. These authors further demonstrated that H_2O_2 stimulated ceramide synthase but not SMase activity, and demonstrated that inhibition of ceramide synthase prevented oxidant-induced ceramide production, DNA damage and cell death. This report provides evidence that ceramide synthase activation by oxidative stress leads to ceramide-mediated injury of renal epithelial tubule cells.

A role for ceramidases during oxidant exposure may also be critical as suggested recently.[96] These investigators demonstrated that exogenously added SMase significantly induced SM hydrolysis that resulted in only a modest ceramide increase in a human proximal tubule HK-2 cell line. This indicated that the vast majority of the generated ceramide was rapidly catabolized (presumably by ceramidases), and underscored the authors' previous conclusion that "the ceramide increments after acute tubular injury must stem, at least in part, from decreased ceramide catabolism, and not simply SM breakdown".[96,97] Therefore, activation of a SMase by it self may not be sufficient for cell death induction during oxidative stress; inhibition of ceramide catabolism (presumably by ceramidases) may also be required.

11.6 Ceramide and Nitric Oxide

Nitric oxide (NO) is a short-lived free radical gas described as a cytotoxic agent in several signaling pathways. Although NO can mediate apoptosis in various cell systems,[98] its mechanism of action is not completely understood. Some studies suggest that ceramide and NO interact to mediate cell death. Chronic exposure of renal mesangial or glomerular endothelial cells to NO donors resulted in a dose-dependent increase in ceramide levels and in the activation of both acidic and neutral Smases.[99,100] In contrast, acidic and neutral ceramidases, the ceramide-metabolizing enzymes, were shown to be inhibited by NO.

In addition to its role as a second messenger in apoptosis, NO, under different conditions, can also protect cells against apoptosis.[101] For example, in human monocytic cells, exogenous NO protects from cell death by inhibiting TNFα-induced TRADD recruitment, caspase-8 activity, and ceramide generation.[102] Likewise, NO inhibits $p75^{NTR}$-induced apoptosis in neuroblastoma cells,[103] but the protective role of NO occurs downstream of ceramide accumulation. Finally, recent studies suggest that NO can act both upstream and downstream of ceramide generation.[104] Other reports describe NO production as a committed step in ceramide signaling. For example, cell-permeable ceramide potentiated the effects of TNFα on NO production and on the inducible-NO synthase expression in glioma cells, rat primary astrocytes and murine macrophages, and in smooth muscle cells, NO production was induced following treatments with exogenous Smase.[105-107] Therefore, although more work is needed to elucidate how the NO system and the ceramide pathway are connected in apoptosis signaling, the two are clearly coupled.

11.7 Ceramide Generation by nSMase is Modulated by GSH

Our laboratory demonstrated that ceramide accumulation following H_2O_2 treatment appears to occur via the activation of SMase pathway(s).[12,13] These pathways also respond to TNFα, Fas, and γ-irradiation-initiated apoptosis[58,59,108] wherein ceramide accumulation is concurrent with SM hydrolysis by activated SMases. However, whereas both the membrane-associated neutral and the acidic forms of SMases are activated by TNFα receptors through different domains, we demonstrated that only nSMase, and not aSMase, is affected by exposure to H_2O_2.[13]

The mechanism by which H_2O_2 stimulates SM hydrolysis to ceramide is unknown. We demonstrated that extracellular supplementation of GSH to A549 lung epithelial cells inhibited ceramide production, whereas depletion of intracellular GSH by H_2O_2 or DL-buthionine-[S,R]-sulfoximine (BSO) paralleled increases in ceramide levels and apoptosis induction.[14] When GSH was supplemented extracellularly, the H_2O_2-induced drop in cellular GSH was diminished and both ceramide elevation and apoptosis prevented. These were all specific effects of GSH and not of other thiol-containing molecules. Importantly, aminotriazole (inhibitor of Catalase) mimicked the effects of H_2O_2 treatment, and N-Acetyl Cysteine (NAC) inhibited the effects of intracellularly generated H_2O_2. These results suggested that in lung epithelial cells, H_2O_2 triggers the apoptotic pathway by inducing ceramide generation via depletion of GSH and that elevation of ceramide is sufficient and necessary for inducing apoptosis.

In several systems, ROS production has been shown to play a key and early role in ceramide-mediated apoptosis induced by serum starvation,[14] anthracyclins such as daunorubicin,[109] and cytokines such as TNF.[19] In these systems, generation of ROS precedes ceramide elevation, and GSH depletion is often evident;[19] supplementation with antioxidants such as GSH and NAC inhibits ceramide accumulation and apoptosis. Therefore, it appears that generation of ROS and increases in ceramide are coupled and mediate cell death due to several diverse apoptotic stimuli. However, how GSH regulates ceramide levels is not yet established.

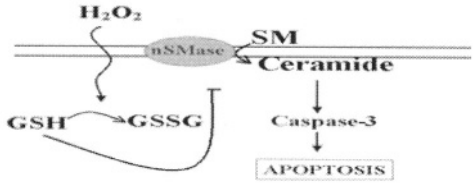

Figure 4: Schematic representation of the role of oxidants (H_2O_2) and antioxidants (GSH) in ceramide generation and apoptosis. GSH inhibits nSMase activity, thus maintaining low ceramide levels. Stress factors (i.e., extracellularly administered H_2O_2) result in ROS-mediated depletion of GSH. Therefore, the redox state of the cell determines the activity of nSMase and the levels of ceramide, thus modulating the apoptotic pathway in lung epithelial cells.

It was recently shown that GSH regulates the neutral Mg^{2+}-dependent nSMase. GSH elicits a direct inhibitory effect on nSMase from blood cells[21,68,110] and on purified nSMase from brain cells.[111] Moreover, decreases in cellular GSH levels induced by TNFα precede activation of nSMase.[21] These are among the observations that led us to propose (Figure 4) that in lung epithelial cells, the plasma membrane-bound nSMase may exist as an inactive form inhibited by high levels of both intra- and extracellular GSH present in epithelial lining fluid (ELF), thus maintaining low levels of ceramide. The inhibition of nSMase would render lung cells less sensitive and less susceptible to oxidants than they are ordinarily exposed to and the threshold for ceramide elevation required for the induction of apoptosis increases. However, when oxidant levels increase and GSH levels drop, the inhibitory effect of GSH on nSMase is overcome, therefore increasing ceramide elevation and the apoptotic pathway is initiated. This hypothesis is supported by our findings that the inhibitory effect of GSH on H_2O_2-induced ceramide production is specific for GSH and not for other thiol-containing molecules and most importantly not for GSSG. Therefore, oxidation of GSH by oxidants renders it incapable of inhibiting ceramide generation. It is interesting that even a short exposure of cells to H_2O_2 for 1 h, followed by incubation in regular media, is sufficient to induce apoptosis. This demonstrates that the events that control the fate of the cells occur within this hour, during which GSH is depleted and ceramide is generated.[14] We also observed that supplementation of GSH shortly before exposure to H_2O_2 was sufficient to inhibit the apoptotic effects of H_2O_2. It appears that providing GSH to replenish the decreased levels of GSH is sufficient to maintain ceramide below the threshold levels, thus preventing apoptosis.

11.8 Ceramide Signaling and Enzymatic Antioxidants

Consistent with the inhibitory effects of GSH on nSMase, [14,21] several studies indicate that antioxidant scavenging proteins can also interfere with ceramide signaling. For example, Mn(III) tetrakis (benzoic acid) porphyrin, a superoxide dismutases (SOD) mimic, inhibits ceramide-induced apoptosis in neuronal cells.[112] Gouaze et al[113] showed that overexpression of the cytosolic/mitochondrial selenium-dependent glutathione peroxidase (GPx) can prevent doxorubicin-induced ROS production, nSMase activation, SM hydrolysis and ceramide generation. In agreement with these observations, cell-permeant ceramide promoted apoptosis in T47D human breast cancer cells, bypassing the doxorubicin-induced SMase activation, but neither GPx overexpression nor treatment with exogenous NAC blocked this event, which indicates that GPx targets an event located upstream to ceramide generation.

11.9 Ceramide Generation is Ipstream of the Executioner Phase of Apoptosis

Few reports[73,113] have examined the temporal placement of ceramide accumulation with respect to caspases in oxidant-induced apoptosis. Caspases are described as the executioners of the program for cell death, but the literature conflicts as to whether ceramide generation triggered by apoptotic inducers precedes caspases or vice versa.[114,115] We demonstrated that ceramide accumulation due to SM hydrolysis (via exposures to either ROS or C6-ceramide), ceramide synthesis or

inhibition of UDP-glucose-ceramide glucosyltransferase, induced caspase-3 activation.[48] Because the cleaved forms of both caspase-3 and PARP were detected subsequent to ceramide increases, this suggests that ceramide accumulation occurs earlier in the apoptotic cascade (Figure 5). These data agree with studies from non-lung cell systems,[116,117] and indicate that ceramide accumulation per se may serve as an initial trigger for apoptosis, though in some systems not sufficient to induce apoptosis without activation of the downstream caspase (s) signal.

When caspase-3 was identified as the mammalian analog of the C. elegans CED-3 gene product, it was suggested that this protease could be a common effector for all apoptotic pathways.[118] However, mice with a homozygous deletion of the caspase-3 gene still had a normal development of all of the organs except for the brain.[119] It was also found that a potent inhibitor of caspase-3 activation was ineffective in preventing ceramide-induced apoptosis in U937 (120). Furthermore, it was shown that caspase-3 deficient MCF7 cells were able to complete nuclear apoptosis in response to ceramide and no PARP cleavage was observed. Together, these studies suggest that caspase-3 is not always essential for apoptosis induction by the ceramide pathway. However, activation of caspase-3 by ceramide and induction of apoptosis were inhibited by overexpression of Bcl2,[91] which indeed has been proposed to act via its inhibition of caspase-3.[118]

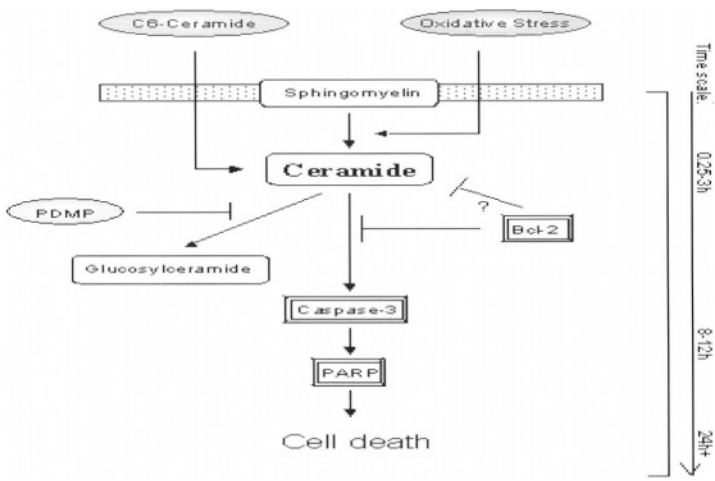

Figure 5: Model for the apoptotic events during ceramide-mediated apoptosis: Caspase-3 is activated following the induction of ceramide accumulation by diverse inducing agents. This results in the cleavage of PARP and other possible target molecules, followed by apoptotic cell death. Bc12 functions to inhibit the activation of caspase-3 and may also prevent ceramide production.

To further investigate where endogenous ceramide generation is located in the apoptotic signaling cascade of lung epithelial cells exposed to oxidative stress, our laboratory[48] studied the role of Bcl2 overexpression on the ceramide-induced

pathway. Some roles of Bcl2 modulating the ceramide pathway have been also suggested. For example, Tepper *et al*[117] demonstrated that Bcl2 overexpression in Jurkat cells reduced ceramide accumulation induced by CD95. On the other hand, Jaffrezou *et al*[121] showed that overexpression of Bcl2 in HL60 cells had no effect on ceramide generation induced by C6-ceramide. Only a few studies reported that Bcl2 could regulate ceramide generation,[91,122] whereas most studies suggested that Bcl2 blocks apoptosis but not ceramide generation.[47,123-125] We have demonstrated that Bcl2 overexpression protects airway epithelial cells against H_2O_2 and C6-ceramide-induced caspase-3 activation and cell death;[48] Bcl2 also inhibited ceramide generation in response to inducers of apoptosis and reduced basal cellular levels of ceramide. Thus, Bcl2 may exert its anti-apoptotic effects via targeting the ceramide pathway (Figure 5). Nevertheless, additional studies are needed to determine whether Bcl2 prevents caspase activation directly or by the inhibition of ceramide generation.

11.10 Ceramide Generates Reactive Oxidants: The Mitochondria Connection

Ceramide induces ROS production in intact mitochondria[16] and in cells,[126] and recent studies have begun to unravel the intimate connections between mitochondrial involvement in apoptosis and the ceramide pathway, including the determination of mitochondrial-specific actions of ceramide. These studies point to ROS generated in the mitochondrial respiratory chain as early mediators of ceramide-induced apoptosis, suggesting that coupling between oxidative stress and ceramide production is bi-directional: not only oxidants activate ceramide production, but ceramide may also induce generation of reactive oxidants.

Because mitochondria have a central role in the control of cell survival and death, the question arose as to whether the ceramide pathway and mitochondrial processes were coupled, and whether there was a mitochondrial pool of ceramide that is directly involved in the progression of the release of cytochrome C and other proteins, which lead to the activation of effector caspases and apoptosis execution.

These questions were addressed when it was reported that ceramide accumulation was detected in mitochondria,[127] and when the addition of exogenous ceramide to purified mitochondria was shown to inhibit the respiratory chain [128] generate ROS and release cytochrome C.[16,128-132] It is, therefore, possible that a mitochondrial pool of ceramide is involved in these processes.

Moreover, several activities of the ceramide-generating machinery appear to reside in the mitochondria, which supports the existence of a pool of SM in mitochondria.[82,83] For example, a ceramide synthase was partially purified from bovine liver mitochondria,[133] and several enzyme activities such as SMase and SM-synthase were identified in mitochondria-associated membranes. The expression of bacterial SMase in mitochondria, but not other subcellular compartments, resulted in induction of apoptosis[83] and points to a role for endogenous mitochondrial ceramide in regulating apoptosis. The recent identification and cloning of a mitochondrial ceramidase,[134] and its mitochondrial localization, has substantiated the existence of mitochondrial pathways of ceramide metabolism that may play a key role in mitochondrial functions and in the regulation of apoptosis.[82]

11.11 Diseases Related to Ceramide Signaling

Reactive oxidant-ceramide coupling appears to play a role in many different diseases such as Macular Degeneration,[135] diseases of the nervous system,[136-138] vascular diseases, insulin resistance and cancer. For the sake of space, we will focus only on some studies related to diseases involving ceramide signaling such as vascular disease, insulin resistance and multi-drug resistance in cancer.

11.11.1 Vasodilatation, Oxidative Stress and Ceramide Generation.

An understanding of how oxidant and ceramide coupling participates in vascular disease is emerging.[139] For example, recent studies showed that ceramide stimulates the production of $O_2^{\cdot-}$ in vascular cells.[140-142] Since $O_2^{\cdot-}$ can interact with NO and thereby decrease NO within endothelial cells,[143,144] it is possible that ceramide-stimulated $O_2^{\cdot-}$ production in the vascular endothelium depletes NO resulting in impairment of endothelium-dependent vasodilatation in the coronary circulation.[145]

It was also shown that NO can induce ceramide production in glomerular mesangial and endothelial cells and that ratio of NO to $O_2^{\cdot-}$ may determine whether cells live or die. Exposure of glomerular endothelial and mesangial cells to either NO donors or superoxide-generating substances led to a sustained ceramide formation that paralleled the induction of apoptosis in both cell types. Co-incubation of endothelial cells with NO and superoxide led to the generation of peroxynitrite and caused a synergistic enhancement of ceramide generation and apoptosis, but co-stimulation with superoxide neutralized not only NO-induced apoptosis but also NO-induced ceramide formation in mesangial cells, although O2- alone triggered ceramide formation and cell death. Furthermore, exposure of endothelial cells to glucose oxidase, which generates H_2O_2, or to exogenous H_2O_2, also showed a dose-dependently increased ceramide formation and apoptosis, although to a lesser extent than exposure to superoxide. Taken together this suggests that ceramide represents an important mediator of reactive oxygen and nitrogen species-triggered cell responses. There also seems to be cell type-specific protective mechanisms that critically depend on a fine-tuned redox balance between reactive nitrogen and oxygen species that determines whether a cell undergoes apoptosis or survives when exposed to oxidative and/or nitrosative stress conditions.[146]

11.11.2 Restenosis and Ceramide Generation

Strategic elevation of cellular ceramide is often used for therapies that aim to control growth or advance apoptosis. On the other hand, agents that reduce ceramide or elevate S1P tend to inhibit apoptosis and enhance proliferation. One remarkable demonstration of the first approach is presented in the work of Kestere *et al* coworkers: they found that ceramide analogs applied directly to damaged arteries can be strongly antiproliferative.[147] Indeed, neointimal hyperplasia of vascular smooth muscle cells and secondary occlusion of coronary arteries, the cause of restenosis after balloon angioplasty or stenting, affects nearly 20% of the 1.5 million patients who undergo coronary angioplasty yearly. Proliferation of cultured vascular smooth muscle cells appears to involve the extracellular signal-regulated kinase

(ERK) and Akt kinase cascades, which can be inhibited by ceramide.[148] In rabbits, C_6-ceramide-coated balloon catheters prevent stretch-induced neointimal hyperplasia in carotid arteries[147] by inactivating ERK and Akt signaling and thereby inducing cell cycle arrest in stretch-injured vascular smooth muscle cells.[148]

11.11.3 Insulin Resistance, Oxidative Stress and Ceramide Generation

Despite enormous effort, the mechanism of insulin resistance that accompanies Type II diabetes remains elusive. Oxidative stress and ceramide have gained considerable attention for their potential roles in contributing to impaired insulin responsiveness.[149] Oxidant stress is directly correlated with metabolic control in patients with Type II diabetes; it is thought that hyperglycemia compromises endogenous antioxidants, which results in the enhanced generation of free radicals.[150,151] It has also been proposed that it is the insulin resistance and compensatory hyperinsulinemia that leads to increased oxidative stress.[152] Ceriello et al observed that insulin stimulates the production of H_2O_2 and proposed that a deleterious cycle exists where hyperglycemia and hyperinsulinemia leads to free radical production, which further impairs insulin action.[150] Studies with diabetic patients and animals indicate that disrupting such a cycle with anti-oxidative therapy improves metabolic parameters[149,153] but these studies have not shed light on the mechanism(s) by which oxidative stress leads to insulin resistance. A number of reports have demonstrated that ceramide inhibits insulin signaling in cultured muscle and fat.[154-157] In addition, ceramide levels have been reported to be elevated in the muscles tissues of genetically obese insulin-resistant rats.[158] Thus, it is possible that oxidative stress and ceramide coupling plays a role in insulin resistance.

Most of the work investigating the effects of oxidative stress and ceramide on insulin signaling was conducted with 3T3-L1 adipocytes. Rudich et al demonstrated that extended exposure to oxidative stress inhibited glucose transporter-4 (GLUT-4) translocation due to insulin in 3T3-L1 adipocytes. Further work by the same group provided evidence that the effects of oxidative stress on insulin responses were due to compromised phosphatidylinositol (PI)-3 kinase activation, insulin receptor substrate (IRS)-l redistribution and Akt activation.[156,157] Anti-oxidants such as lipoic acid can reverse oxidant-impaired insulin responsive.[159,160] On the other hand, Long et al demonstrated that exposure to membrane-permeable ceramide decreases GLUT-4 mRNA levels[161] in 3T3-L1 adipocytes, and Peraldi et al showed that treatment with ceramide or with bacterial SMase inhibited insulin-stimulated IR and IRS-1 tyrosine phosphorylation.[162] Subsequent work by other groups confirmed that exogenous ceramide inhibited insulin-induced glucose uptake. Recent work by Summers et al showed that membrane-permeable ceramide suppressed Akt without affecting IRS-1 tyrosine phosphorylation or IRS-1 associated PI3 kinase activity.[163] Muscle cells exposed to oxidative stress or ceramide exhibit similar impairments in insulin responsiveness as adipocytes.[154,155] Unfortunately, there are no reports in the literature that have explored whether oxidative stress stimulates ceramide production in muscle and fat cells.

A deleterious cycle likely exists between proinflammatory cytokines, oxidative stress and ceramide in insulin resistance. TNFα is reportedly elevated in the muscle and adipose tissue of insulin resistant humans and animals and it has been demonstrated to increase endogenous ceramide and impair insulin signaling in

cultured muscle and fat cells.[164,165] On the other hand, TNFα can also modulate the cellular redox status of muscle and adipocytes.[166,167] Such a loop makes determining the inciting factor in insulin resistance difficult to determine. Nevertheless, because TNFα and oxidative stress stimulate endogenous ceramide accumulation in a variety of different cell types, ceramide accumulation may very well act as the common pathway for those factors that leads to impaired insulin signaling. Thus, preventing ceramide accumulation may be a useful therapeutic strategy for improving insulin sensitivity in Type II diabetes.

11.11.4 Drug Resistance, Oxidative Stress and Ceramide Generation

Cancer cells develop multiple mechanisms to become resistant to chemotherapeutic agents.[168,169] Multi drug resistance may be caused by diverse mechanisms such as activation of efflux pumps, which lower drug levels within the cells[170,171] or modifications of glutathione metabolism, which may protect against oxidant stress.[172] A number of studies suggest that dysfunction of ceramide metabolism may contribute to multi drug resistance.[61,173,174] For example, excessive ceramide glycosylation by the enzyme glucosylceramide synthase (GCS) (see Figure 1) has been observed in some multi drug resistant cell lines.[175,176] Since glucosylceramide is a noncytotoxic metabolite of ceramide, this enzymatic reaction may be an important pathway for bypassing apoptosis induced by ceramide.

It was recently suggested that the resistance of cancer cells to drugs or other apoptosis-inducing agents could be reversed by targeting ceramide metabolism.[173] Notably, a number of clinically important chemotherapeutic agents inhibit tumor growth because of their ability to enhance ceramide formation in cancer cells[173] via the activation of various pathways of ceramide metabolism. This in turn can overcome an inhibition of ceramide accumulation, which may be conferred, for example by elevating GCS activity.[174] For example, anthracyclins such as doxorubicin and daunorubicin elevate ceramide levels by activating ceramide synthase as well as Smases.[115,177] Tamoxifen (an estrogen analog used to block estrogen receptor), on the other hand inhibits the conversion of ceramide to glucosylceramide by GCS,[178] thereby increasing cellular ceramide levels. Another chemotherapeutic drug that modulates ceramide metabolism is the synthetic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR), which increases intracellular ceramide levels in diverse cell types.[179] The increase in ceramide by 4-HPR was suggested to involve ceramide synthase since ceramide increase was abrogated by inhibitors of de novo ceramide synthesis.[180] This drug also mediates p53-independent cytotoxiciy and can increase reactive oxygen species and ceramide in solid tumor cell lines.[180]

Modulation of ceramide levels may also augment the efficacy of some cancer treatments.[177] For example, Mehta et al showed that the addition of ceramide boosts taxol-mediated apoptotic death of Tu138 head and neck tumor cells.[181] Selzner *et al* showed that the cellular content of ceramide in human colon cancer is reduced by more than 50% relative to that of healthy colon mucosa.[90] The effective ceramidase inhibitor B13 increases the ceramide content of tumor cells and induces tumor cell apoptosis, without affecting the ceramide level or survival of normal liver cells. B13 also prevents growth of two aggressive human colon cancer cell lines metastatic to

the liver, suggesting that ceramidase inhibition may provide a promising therapeutic strategy for selective toxicity toward malignant but not normal cells.

Interestingly, some of the chemotherapeutic agents that modulate ceramide levels also produce ROS. The anthracyclins, for example, elicit ROS formation[109] that may contribute to anthracyclin cytotoxiciy.[182] Gouaze *et al.*[113] have shown that apoptosis induced by doxorubicin is preceded by ROS production. Treatment of cancer cells with sodium nitroprusside, a nitric oxide donor, was also associated with elevation of ceramide via the activation of nSMase[183] that was followed by apoptosis, establishing a relationship between the ceramide pathway and NO-mediated apoptosis and pointing to a new strategy for chemotherapeutic intervention.

The overall importance of oxidants in cancer treatment was demonstrated by pretreatment of cancer cells with NAC, which resulted in inhibition of both ceramide production and cell death.[109] This suggests that ROS production may be a major constituent in chemotherapeutic agents-induced apoptosis via ceramide generating pathways, and illustrates the concept that cellular antioxidant defense can influence the clinical efficiency of such agents (i.e., anthracyclins) by modulating the coupling of ROS and ceramide.

In summary, recent studies have implied that dysfunction of ceramide metabolism, observed in multi drug resistant cancers, may be overcome by chemotherapeutic agents that modulate the ceramide pathway. The ability of some of these drugs to produce ROS may contribute to their ability to generate ceramide and induce apoptosis. However, for rational advances in chemotherapy to proceed, the exact mechanism and specific targets for ROS in the modulation of ceramide levels in cancer cells that turn drug resistant need to be defined.

11.12 Conclusion

Several lines of evidence indicate that oxidative stress and ceramide generation are intimately coupled in cell signaling: 1. Inducers of apoptosis trigger the generation of both ROS and ceramide; 2. Oxidative stress-induced apoptosis involves the SM-ceramide pathway[3]. Ceramide stimulates ROS production and may act directly on the mitochondrial respiratory chain; 4. Cellular antioxidants regulate ceramide buildup; 5. Growing evidence suggests that the pathologic states of several diseases that are affected by reactive oxidants involve the regulation of the ceramide pathway.

The kinetics of ROS and ceramide generation in cell death show that both occur early in the commitment phase of the apoptotic cascade; ceramide accumulation occurs prior to the execution phase initiated by caspases and may also be upstream of the site where Bcl2 acts. However, more work is needed to determine the exact cause-effect relationships between oxidative stress, ceramide, caspase activation and Bcl2 inhibition.

While it is unequivocal that oxidative stress modulates ceramide production, ROS generation and mitochondrial alterations may also characterize a committed phase in ceramide signaling, indicating that positive feedback may occur between ROS and ceramide generation in cell death. However, many of the links between oxidative stress and ceramide signaling are still correlative and require rigorous molecular and mechanistic studies. The exact molecular mechanisms and the

subcellular localization of oxidative stress and ceramide pathway(s) interactions need to be defined. The molecular identification of SMases, SM-synthase, ceramidases that are modulated by oxidative stress is not complete. Recent studies reported on the cloning of two candidate nSMases,[184] but evidence was provided that one nSMase localizes to the endoplasmic reticulum and functions as a lyso-PAF phospholipase C,[185] whereas the other nSMase localizes to the Golgi and its physiologic substrates were not established. Because enzymatic and sub fractionation studies indicate the presence of a plasma membrane nSMase that is modulated by oxidative stress, effort is still being directed toward isolating and characterizing this SMase from the lung [Goldkorn *et al,* unpublished data]. Similarly, very little is known about the SM-synthase, which also has been difficult to purify and clone.[81] Because most of the key enzymes regulating ceramide metabolism have not been characterized yet there is still a lack of molecular and pharmacological tools to study these pathways and their functions.

On one hand, it is desired to prevent the killing of cells and tissue injury that oxidant-ceramide coupling leads to in diseases such as Asthma or ARDS. In cancer, the opposite effect is sought.

Decoding the exact molecular interactions between oxidative stress and ceramide pathways should lead to new strategies for pharmacological intervention in such diverse diseases.

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