Ceramide: A Novel Second Messenger

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Cellular responses to extracellular signals are mediated by receptors, which are coupled to effector systems through generation of second messengers. Sphingomyelin (SM) pathway is one among such signalling systems, analogous to conventional systems like cAMP, cGMP, etc. Ceramide is generated from sphingomyelin by the action of neutral or acidic sphingomyelinase. Mediators of ceramide include ceramide-activated protein kinase, ceramide-activated protein phosphatase and atypical form of protein kinase Cx. SM pathway induces differentiation, proliferation and apoptosis depending on type of cell and a general outcome of this signalling system is apoptosis. Furthermore, ceramide is believed to be involved in diseases like ischemia/reperfusion injury, insulin resistance, atherogenesis, etc. A better understanding of molecular mechanisms of physiological and pathological characteristics of this signalling system may unveil novel therapeutic targets.

Key Words: Ceramide, Sphingomyelin, Second messenger, Sphingomyelinase

Introduction

Most of the changes in cellular physiology induced by extracellular signals are mediated by receptors. These receptors are coupled to effector system, which include cAMP and phosphoinositide systems. Recent investigations led to the evolution of a number of second messenger systems. Sphingomyelin (SM) pathway is one such with ceramide acting as the key molecule. This is ubiquitous and evolutionarily conserved from yeast to mammals. This signalling system is analogous to conventional cAMP and phosphoinositide pathways. Ceramide plays a pivotal role as a second messenger in this pathway and influences in the regulation of cell growth, differentiation and apoptosis. Ceramide is generated from membrane sphingolipids by the action of sphingomyelinases (SMases). Sphingolipids have a role in response to cell contact, as receptor components, as anchors for protein and markers of tumor progression and cell differentiation (Hannun 1996). Several bacterial haemolysins and cytotoxins have been identified as sphingomyelinases and fungal metabolites like fumonisins effect sphingolipid metabolism (Hannun 1996) indicating the importance of sphingolipids in cell regulation. Hence a better understanding of molecular basis of ceramide actions would yield better therapeutic targets for the study of pathogenesis and progression of various diseases like cancer, HIV infection, neurodegenerative disorders, cardiovascular and autoimmune diseases.

Generation and Metabolism of Ceramide

Ceramide is generated by hydrolysis of sphingomyelin by various sphingomyelinases. SM is preferentially concentrated in the outer leaflet of plasma membrane of most mammalian cells. It consists of a long chain called sphingosine linked to phosphocholine and the amino terminus of sphingosine is connected to a fatty acid by means of an amide bond. Hydrolysis of phosphodiester bond by SMases generates ceramide (Kolesnick & Golde 1994) (figure 1).

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endothelial cells secrete substantial amount of high molecular mass form of acid SMase (Marathe et al. 1998). Studies on foetal bovine serum demonstrated that acidic SMase activity is stimulated by Zn\(^{2+}\) ions (Spence et al. 1989). Secreted form is stimulated by Zn\(^{2+}\), whereas lysosomal form is not. Acidic SMase secretion is increased by cytokine stimulation by increasing synthesis of precursor form by golgi secretory pathway. Acidic environment is not required for the activity of the SMase, but when present, enhances its activity (Schissel et al. 1996).

Neutral SMases (pH 7.4) are yet to be characterised at genetic level, as they are the products of a separate gene. They are generally membrane bound and Mg\(^{2+}\) dependent. A similar cytosolic, Mg\(^{2+}\)-independent isoform has also been described (Okazaki et al. 1994). Alkaline SMase has been detected in intestinal mucosa and is thought to be involved in digestion and mucosal cell proliferation (Mathias et al. 1998).

### Metabolism of Ceramide

Ceramide can be de novo synthesized by condensation of serine and palmitoyl-CoA to 3-oxosphingonine, which is subsequently reduced to sphingosine, to which addition of amide-linked fatty acid by ceramide synthase yields dihydroceramide. It serves as a precursor for ceramide synthesis. Dihydroceramide can be converted to dihydroform of glucosylceramide and sphingomyelin. This route is activated by radiation and requires several hours (Mathias et al. 1998).

Ceramide once formed is converted to respective metabolites. Phosphorylation by ceramide kinase (Kolesnick & Golde 1994) generates ceramide-1-phosphate while deacylation by ceramidases yields sphingosine, which then is phosphorylated to sphingosine-1-phosphate. Alternately ceramide is converted to sphingomyelin by sphingomyelin synthase.

### Mechanisms of Ceramide Action

Biologically relevant and direct targets of ceramide are: protein phosphatase (CAPP), protein kinase (CAPK) and Protein kinase C ζ (PKC ζ) (an atypical isoform of protein kinase C).

**Ceramide-activated protein phosphatase:** CAPP belongs to protein phosphatase 2A series of a family
of serine/threonine phosphatases and is potently inhibited by okadaic acid (Hannun 1996). It is present in cytosol and activated by ceramide but not by dihydroceramide (Kolesnick & Golde 1994). Effects of ceramide mediated by this enzyme are down regulation of c-myc protooncogene expression in mammalian cell (Moon & Woo 1998). Further studies are required for the identification of physiological substrates of CAPP.

Ceramide-activated protein kinase: Ceramide-activated protein kinase, which is a member of a family of proline-directed serine/threonine protein kinases that recognize substrates containing minimal sequence X-Ser-/Thr-Pro-X (Kolesnick & Golde 1994). It is exclusively bound to membrane, and autophosphorylates on serine residues.

Ceramide-activated protein kinase C \( \zeta \): PKC \( \zeta \) is an atypical PKC isoform, which is insensitive to diacylglycerol and phorbol esters but responsive to ceramide (van Blitterswijk 1998). PKC \( \zeta \) may play a role in TNF-induced activation of nuclear factor kB (NFkB) in some cells.

**Biological Implications of Ceramide**

**Apoptosis**

In regular physiological conditions cells die as a part of the enactment of a developmental programme (Glucksmann 1951). Unique characters of this developmentally regulated cell-death has been encapsulated by the term programmed cell death (Lockshin & Beaulolton 1974), consists of distinct set of structural changes. This co-ordinated structural changes involve interaction between initiating stimuli and factors that determine susceptibility of cells to activate the terminal effector events. Ceramide is thought to act as a second messenger in activating apoptotic cascade (Dawson et al. 1998). Diverse cytokine receptors and environmental stresses utilise ceramide to signal apoptosis (Friedmann et al. 1997). Different stimuli which cause apoptosis through ceramide mediation are TNF-\( \alpha \) (Scurlock & Dawson 1999), ionising radiation, chemotherapeutic agents (Goswami & Dawson 2000), photodynamic treatment (Duska et al. 1998) and hyperglycemia (Moley 1998).

Ceramide mediated pathway is considered to be an upstream mechanism for apoptosis by various cell surface receptors and environmental stressers. In several systems ceramide links to stress-activated protein kinase/c-jun kinase (JNK) cascade to signal apoptosis (Friedmann et al. 1997). Many of the initiating stimuli generate ceramide from SMase pathway (Dawson et al. 1998). In contrast, daunorubicin-induced apoptosis involves generation of ceramide from de novo synthesis by ceramide synthase (Bose et al. 1995). The critical role of acidic SMase in generating ceramide was confirmed from studies using genetic models of acidic SMase activity (Santana et al. 1996). For instance acidic SMase knock out mice were resistant to apoptosis mediated by ionising radiation or lipopolysacharide (LPS)/TNF-\( \alpha \). Similarly lymphoblasts from patients with Niemann-Pick disease (NP disease), which are deficient in acid sphingomyelinase failed to respond to ionising radiation. But these abnormalities were reversible upon restoration of acid sphingomyelinase activity by retroviral transfer of human acidic SMase cDNA. Conflicting data for the role of acidic SMase is reported as CD95 (Fas/Apo-1)-induced apoptosis occurred in the absence of acidic SMase (De Cock et al. 1998).

Terminal effector points of ceramide mediated signalling involves activation of interleukin 1\( \beta \) converting enzyme (ICE) family of proteases known as caspases (Smyth et al. 1996). Ceramide-induced apoptosis reported to be inhibited by benzylxycarbonyl-val-alal-DLASP-flourumethylketone (Perry et al. 1998), an inhibitor of caspases. Several targets for apoptotic action of ICE/Ced-3 proteases include poly(ADP-ribose) polymerase (PARP), involved in DNA damage repair. Other targets include fodrin, terminin, and protein component of U1 small nuclear protein. Ceramide mediated apoptosis involves proteolytic cleavage of PARP by protein phosphatase, a protein directed kinase and an isofom of PKC (\( \zeta \)) as pretreatment with okadaic acid, an inhibitor of protein phosphatase reverses the apoptosis (Smyth et al. 1996).

Apoptogenic properties of ceramide are thought to be mediated by its effect on mitochondrial functions. Ceramide and cell permeable analogs like \( \zeta \)-ceramide and \( \zeta \)-ceramide and to a lesser extent \( \zeta \)-dihydroceramide induce release of cytochrome-c from isolated rat liver mitochondria (Pedram et al. 1999), which was inhibited by BCl\(_2\). Release of
cytochrome-c takes place in its oxidised, but not reduced, form. Upon its release mitochondrial oxygen consumption, transmembrane potential as Ca\(^2+\) retention are diminished. Another distinct mitochondrial event involves generation of reactive oxygen species in ceramide-induced apoptosis (Espostis & Lenna 1998). The site of action of ceramide was shown to be the at respiratory complex III (Gudz et al. 1997) since inhibition of electron transport thorough complex I and II, blocked ceramide action. Additional role during apoptosis at the level of mitochondria include permeability transition associated with opening of large pores in mitochondrial inner membrane leading to diffusion of substances of molecular mass less than 1.5kDa. This transition leads to release of proteases, which amplify apoptotic signal.

**Immune System**

Ceramide serves as a second messenger for TNF-\(\alpha\), IL-1 (Kolesnick & Golde 1994), interferon-\(\gamma\) (Kim et al. 1991) and various other immune responders. TNF-\(\alpha\) and IFN-\(\alpha\) induce monocyte differentiation through sphingomyelin degradation (Kolesnick & Golde 1994). Evidence was provided that ceramide mediated, TNF-\(\alpha\)-induced down-regulation of \(c\)-\(myc\), may be important for cessation of proliferation during terminal differentiation. Similarly effect of IL-1 on inducing cyclooxygenase (COX) gene expression is mediated by ceramide. Sphingomyelinase, but not phospholipase A\(_2\), C, and D, recapitulated the effects of IL-1 in stimulating IL-2 secretion (Kolesnick & Golde 1994). Ceramide potentiates the action of IL-1 in stimulating the synthesis of prostaglandinE\(_2\) (PGE\(_2\)), by reducing the time required to synthesise the COX-2 mRNA and accumulation of COX-2 protein.

Ceramide signalling also contributes to a variety of inflammatory responses. Conflicting reports are also presented (Carl & Edward 1998). Exogenous ceramide inhibited both respiratory burst and antibody dependent phagocytosis. It also inhibited phorbol ester and TNF-\(\alpha\) induced superoxide release from neutrophils, thereby attenuating inflammatory response of neutrophils (Mathias et al. 1998). In some cell types it also potentiated the release of arachidonic acid by other agents (Takashii et al. 1999). Infections by some bacterial pathogens are mediated by ceramide generation (Mathias et al. 1998). Invasion of epithelial cells and fibroblasts by Niesseria gonorrhoeae is mediated by generation of ceramide by acid SMase (Sandhoff & Kolter 1997).

In HIV infected patients disease progression is characterised by T cell depletion by apoptosis. TNF-\(\alpha\) is a potent activator of HIV proviral transfection in T lymphocytes, owing to activation of NF-kB (Kolesnick & Golde 1994). Signal transduction mechanisms involves acidic SMase, sphingomyelin breakdown and ceramide production. Circulatory population of CD4\(^+\) and CD8\(^+\) T cells from HIV infected patients display large increase in ceramide content and apoptosis to compared with normal population (Mathias et al. 1998). L-carnitine, which reduces acidic SMase activity, significantly reduced ceramide levels and decreased the apoptosis of CD4\(^+\) cells. Moretti et al. (1998) conducted a pilot study in which they administered L-carnitine (intravenously) daily (6g) for 4 months. They reported increased CD4\(^+\) counts and a significant drop in frequency of apoptosis in CD4\(^+\) (greatly) and CD8\(^+\) lymphocytes (lesser extent) (Moretti et al 1998). Hence detailed evaluation of ceramide signaling in uncontrolled apoptosis of T-lymphocytes in case of HIV patients might lead to a novel class of therapeutic agents, which can control HIV infection.

**Endocrine System**

Ceramide mediates the effect of various cytokines on endocrine functions. Adipocytes considered as passive participants in energy storage and release, are now considered to be key regulatory systems for maintenance of energy homeostasis. Adipocytes are capable of secreting cytokines like TNF-\(\alpha\) and ‘ob’ gene. TNF-\(\alpha\) mediates insulin resistance in obese states. Animal studies have suggested that TNF-\(\alpha\) inhibits insulin stimulated glucose uptake in adipose tissue and skeletal muscle. TNF-\(\alpha\) may decrease insulin-stimulated autophosphorylation of insulin receptor and phosphorylation of insulin receptor substrate. Alternatively in 3T\(_3\)-L\(_1\) adipocytes, TNF-\(\alpha\) down regulates the glucose transporter GLUT\(_4\) and attenuates insulin uptake (Sheria & Phylip 1996). Ceramide, like TNF-\(\alpha\), stimulates serine phosphorylation of insulin receptor and/or tyrosine phosphorylation of insulin receptor substrate-1. Further, ceramide mediates the effects
of TNF-α-induced inhibition of protein phosphatase-1 activation, thereby attenuating insulin stimulated glucose uptake, glycogen synthase activity and glycogen synthesis (Begum et al. 1996). Scott et al. (1998) reported ceramide as antagonist for insulin dependent physiological events such as peripheral activation of glucose transporter. It was reported that C2-ceramide inhibited insulin stimulated glucose transport in 3T3-L1 adipocytes (David et al. 1998). C2-ceramide inhibited phosphorylation and activation of Akt, a molecule proposed to mediate the effects of insulin (Scott et al. 1998).

Ceramide signalling is also involved in the condition of diabetes, by affecting pancreatic β-cell function. In the pancreas, macrophages infiltrating the islet cells produce IL-1β, which is believed to play a role in triggering the onset of type-1 diabetes. Ceramide mimicked the effects of IL-1β on this system causing inhibition of insulin production perhaps by activating protein phosphatase 2A (Kowluru & Metz 1997). Further, the lipoapoptosis of β cells was observed in fat laden islets of obese fa/fa zucker diabetic rats, which is thought to be the result of over production of ceramide from fatty acids as serine palmitoyl transferase inhibitors inhibited lipoapoptosis (Michio et al. 1998).

In the ovary, ceramide mediates the effects of various cytokines on regular cyclic process. TNF-α released by macrophages, oocytes and other follicular cells provides physiologic stimulus for ceramide generation and apoptosis of granulosa cells during follicle artesia (Wilty et al. 1996). Ceramide antagonizes the effects of follicle stimulating hormone in isolated follicles, similar to TNF-α, inducing apoptosis of follicular cells (Kaipia et al. 1996). Further, ceramide mediates the effects of antiovulatory state associated with adrenal hyperactivity or excess of glucocorticoids (Minoru et al. 1999). Ovulation is considered to be cyclic inflammatory process where IL-1 induction and increased biosynthesis of prostanoids may feature prominently. Hence antiovulatory effects of glucocorticoids may be due to interference with ovarian prostanoid biosynthesis. Ceramide analogs proved to be as effective as dexamethasone in suppressing IL-1 induced expression and ovarian prostaglandin endoperoxide synthase activity.

**Cardiovascular System**

Now a days diseases of cardiovascular system such as hypertension and atherosclerosis are more prevalent. Research is focussed on physiological factors responsible for and progression of these diseases. Identification of cellular mechanisms that regulate vascular contractility and cell proliferation are crucial for the development of better therapeutic interventions to combat these diseases. Ceramide is reported to be a novel-signalling pathway to target these diseases.

In the vascular system ceramide regulates apoptosis and inflammation. SMase activation is associated with generation of ceramide is implicated in the pathogenesis of several diseases like atherosclerosis, reperfusion injury, vascular contractility and ischemic heart diseases.

Blood platelets, which are frequently used for examining signal transduction system lack nucleus and therefore addition of ceramide to platelets can be used for evaluating actions of lipids other than those obtained in proliferative cells. Activation of platelets by thrombin causes platelets to release sphingosine-1-phosphate and sphingosine. Sphingosine-1-phosphate is also known to potentiate platelet aggregation. Thus spingolipids serve as second messenger during platelet activation. Conflicting reports over the role of ceramides in platelet aggregation are also reported (Carl & Edward 1998). Exogenous addition of ceramide analogs enhances arachidonic acid release by increasing susceptibility of substrate phospholipids to PLA2 (Hashizume et al. 1999). Ceramide does not induce any response on its own. Ceramide triggers PLC activation synergistically with thrombin and potentiates sequential PKC-MAPK cascade, resulting in enhancement of arachidonic acid release. Further platelet activating factor (PAF) also has a modulatory action on sphingoid metabolism as ceramide generation is blocked by PAF receptor antagonist (Edwardo et al. 1999). Sphingosine activates phosphoinositol-4 kinase (PI4 kinase) and enhances agonist induced PL-C activation leading to platelet activation. But the effects of cell permeable analogs depend on N-acyl chain length. Cell permeable analogs like C2-ceramide inhibited platelet aggregation in dose dependent manner but C8 ceramides markedly
enhanced platelet aggregation induced by thrombin (Tsutomu et al. 1998).

Ceramide has also been implicated in aspects of TNF-α action on endothelial cells. TNF-α induces release of plasminogen activator inhibitor (PAI) from human umbilical vein endothelial cells by an increase in the activity of acidic SMase activity (Shinju et al. 1998). Cell permeable C2-ceramide, but not dihydroceramide, enhances the level of PAI-1. Treatment of human umbilical vein cells with high doses of SMases, induced expression of inflammatory cytokines, IL-6, IL-8 and adhesion molecules like E-selectin (Masamune et al. 1996). Thus ceramide may also act as a signal for inflammatory mediators in endothelial cells (Modur et al. 1996).

Ceramide also contributes to the development of atherosclerotic or thrombotic diseases. Atherosclerosis is a disease process characterised by aggregation of lipoproteins in the arterial wall, foam cell formation and smooth muscle proliferation. Smooth muscle cell proliferation is a hallmark in the pathogenesis of atherosclerotic lesion. The atherosclerotic lesion most probably develops through a number of cellular events which includes all vascular cell types and synthesis of extracellular proteins, cell proliferation, differentiation and death. Exogenous sphingolipids mediate various biological effects like apoptosis and mitogenesis. Oxidised LDL, growth factors or cytokines which activate intracellular signalling pathways leading to vascular cell modifications can stimulate sphingomyelin hydrolysis and generation of ceramide (Augé et al. 2000). Sphingolipids and sphingolipid metabolizing enzymes may also play an important role in atherogenesis. When endothelial cells, and attached monocytes/macrophages are activated they generate free radicals and oxidise LDL. Oxidised lipoproteins induce activation of neutral SMase (Schissel et al. 1998) and SM hydrolysis leading to ceramide production. Marathe et al. (1998) reported the presence of SMase in atherosclerotic lesions whose activity may promote atherosclerosis by enhancing subendothelial LDL retention and aggregation. SMases present in atherosclerotic lesions may promote atherosclerosis by enhancing sub endothelial LDL retention and aggregation (Marathe et al. 1999). These activities were mimicked by ceramide generated in plasma membrane by bacterial SMase treatment and by the addition of exogenous ceramide.

Other targets of ceramide include vascular smooth muscle. Pin-Lan et al. (1999) reported the vasoconstrictor effect of ceramide on coronary arterial smooth muscle cells. In vascular smooth muscle cells ceramide is generated from acidic SMase. Exogenous addition of C2-ceramide in concentration dependent manner decreased the K_{ca} activity in vascular smooth muscle cells (Pin-Lan et al. 1999). Further Douglas et al. (1998) reported endothelium dependent ceramide induced vasodilation in intact rat thoracic aortic rings. Similar reports were presented in which ceramide acts as a second messenger in relaxing the phenylephrine preconstricted aortic rings from Sprague Dawley rats (Jong-shiaw et al. 1999). Further levels of ceramide were found to be elevated in cytokine, TNF-α induced apoptosis in cardiac myocytes. Elevated levels of TNF-α were found in variety of clinical conditions like sepsis, ischemic myocardial disorders associated with cardiac cell death. Hence ceramide mediated apoptosis may play a beneficial role in limiting area of cardiac cell involvement as a constituent of myocardial infarction (Kevin et al. 1996).

Ceramide also play a role in ischemic reperfusion injury in cardiac myocytes. Elevated levels of oxygen free radicals in case of reperfusion is well documented. Targets of these radicals include extracellularly activated and stress-activated kinases. One of the earliest response of these activation include activation of neutral SMase and accumulation of ceramide, whose effects are quenched by pretreatment with antioxidants (Hemadez et al. 2000).

**Central Nervous System**

Ceramide is considered as a second messenger in cellular growth, differentiation and cell death. Similarly ceramide is required for cell survival and dendritic outgrowth of cerebellar purkinje neurons. Further ceramide regulates the fate of hippocampal neurons in a concentration dependent and developmental stage dependent manner. It plays the growth supportive role in hippocampal culture neurons. But at higher concentrations ceramide caused retraction of dendrites followed by cell
death. In case of mature hippocampal neurons ceramide caused non-necrotic cell death even at low concentrations (Mitom et al. 1998). Shigeki et al. (1998) reported the requirement of ceramide for normal growth of cultured cerebellar purkinje cells. Further, inhibition of ceramide generation by serine palmitoyltransferase inhibitor resulted in decrease in cell survival, accompanied by apoptotic cell death. They reported the synergistic role of ceramide with neutrophils in supporting purkinje cell survival (Shigeki et al. 1998). Andreas et al (1997) reported three distinct roles of ceramide at different stages of neuronal growth: 1) During axonal growth ceramide must be metabolised to glucosylceramide to sustain growth. 2) Formation of minor process can be stimulated by short acyl chain analogs and 3) During both these processes, incubation with high concentration of ceramide, induces apoptosis. Hence ceramide in a concentration and developmental stage dependent manner effects cellular growth and differentiation in case of cultural cerebellar neurons.

Ceramide is also suggested to play a role in neurotransmitter release as the enzyme ceramide kinase is copurified with synaptic vesicles. Ceramide kinase is activated by micro molar concentration of Ca²⁺ leading to phophorylation of ceramide, which is thought to play a role in neurotransmitter release (Bajjalieh et al. 1989). Ceramide induces long term-depressed modulation of synaptic transmission mediated by ionotropic glutamate receptors in the hippocampus through activation of postsynaptic protein phosphatase 1 and 2A. This long term depressed modulation of ceramide on ionotropic glutamate receptor function may be important in various physiological and pathological conditions like excitotoxicity (Yang 2000).

**Miscellaneous**

In case of synovial system, cell surface receptors for TNF-α and IL-1 and Fas are present and the effects of these were mediated by ceramide leading to apoptosis of synovial cells developing rheumatoid arthritis (Noboru et al. 1998). Hence ceramide signalling can function as a novel therapeutic target for the treatment of rheumatoid arthritis.

**Conclusions**

Ceramide has been considered to affect major cellular processes like cell death, differentiation and growth arrest. The range of effects depends on type of cells involved. This high level of regulation may provide better opportunities for pharmacological intervention. Interruption of apoptotic signals may prevent inadvertent death of normal tissues surrounding tumours. With activation of ceramide pathway we can increase the susceptibility of tissues to apoptogenic signals induced by radiation therapy, chemotherapy, etc. Inhibition of ceramide mediated actions like activation of atherosclerotic lesion formation and altered plasma lipoprotein levels may provide better insights for combating atherosclerosis. As ceramide also participates in the death of cardiac myocytes by ischemic reperfusion injury, insulin resistance, HIV infection, and stress-activated neuronal death, a better understanding of molecular mechanisms may unveil better molecular targets for pharmacological intervention.

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