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CERAMIDE DIFFERENTIALLY REGULATES PROTEIN KINASE C/ MITOGEN ACTIVATED PROTEIN KINASE PATHWAYS: IMPLICATIONS FOR GROWTH ARREST

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ABSTRACT

Ceramide, a sphingomyelin-derived second messenger, has been shown to stimulate signaling pathways that lead to growth arrest, differentiation or apoptosis. However, the mechanisms by which ceramide induces these cellular phenotypes are unclear at this time. Our studies establish a new paradigm for actions of lipid-derived second messengers: coordinating assembly of multi-factoral signaling complexes.

The Stress-Activated Protein Kinase (SAPK) pathway has been implicated in cell growth arrest and/or apoptosis. In addition, studies have linked ceramide with activation of the SAPK cascade. However, the mechanism by which ceramide leads to activation of this cascade is unclear at this time. Our studies demonstrate that ceramide activates the SAPK cascade via direct activation of Protein Kinase C zeta (PKCζ) in human embryonic kidney cells (HEK 293). Upon activation by ceramide, PKCζ forms a signaling complex with upstream components of the SAPK cascade, including MEKK1 and SEK1. These studies demonstrate a novel mechanism by which ceramide activates the SAPK pathway to induce cell cycle arrest.

A parallel pathway to the SAPK cascade is the Extracellular signal-Regulated Kinase (ERK) cascade. We and others have shown that the inhibitory action of ceramide on cell growth involves inhibition of the ERK pathway. Therefore, we investigated the mechanism by which ceramide inhibits this pathway. Our studies demonstrate that ceramide inhibits PKCε activity and

subsequent interaction with upstream components of the ERK cascade. These studies characterize a mechanism by which ceramide induces growth arrest through inhibition of the ERK cascade.

Another signaling pathway critical in cell survival and proliferation is the PI_3K/Akt1 cascade. Recent studies have demonstrated that ceramide inhibits Akt1. However, the mechanism of Akt1 inhibition by ceramide is unclear. Therefore, we investigated the role of PKCζ in ceramide-mediated inhibition of the Akt1 cascade. Our studies revealed that inhibition of Akt1 by ceramide is PKCζ-dependent.

Collectively, our studies demonstrate several complimentary mechanisms by which ceramide induces cell growth arrest. The ability of ceramide to induce growth arrest, without inducing significant apoptosis or necrosis, may be of therapeutic value in the prevention or control of cell proliferation during inflammatory renal and vascular diseases.

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ABBREVIATIONS

α	Alpha
ATP	Adenosine 5'-triphosphate
β	Beta
°C	Degrees Celsius
CAPK	Ceramide-Activated Protein Kinase
CAPP	Ceramide-Activated Protein Phosphatase
C_6-Cer	C_6-Ceramide (N-Hexanoyl-D-erythro-Sphingosine)
$C_{18:1}$-Cer	$C_{18:1}$-Ceramide (N-Oleoyl-D-erythro-Sphingosine)
Co-IP	Co-immunoprecipitation
DAG	Diacylglycerol (1,2-Diolein)
δ	Delta
DH	Dihydro-C_6-Ceramide
DTT	Dithiothreotol
ECL®	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol bis(b-aminoethyl ether)-N, N, N', N'-tetraacetic acid
ε	Epsilon
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
HEK 293	Human Embryonic Kidney cell line 293
HCl	Hydrochloric Acid

HRP	Horseradish Peroxidase
IGF	Insulin-like Growth Factor 1
IL-1	Interleukin-1
IP	Immunoprecipitate
K_A	Kinetics of Association
K_D	Kinetics of Dissociation
KSR	Kinase Suppressor of Ras
MAPK	Mitogen Activated Protein Kinase
μCi	microcurie
μg	micrograms
μM	micromolar
mg	milligrams
mM	millimolar
M	Molar
NaCl	Sodium Chloride
NaF	Sodium Fluoride
ng	nanograms
nM	nanomolar
$NaVO_4$	Sodium Orthovanadate
OAG	1-Oleoyl-2-Acetyl Glycerol
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen

PDGF	Platelet-Derived Growth Factor
PDK1	Phosphoinositide(3,4,5)P_3-Dependent 3-Kinase 1
PKC	Protein Kinase C
pH	Hydrogen ion concentration
PH	Pleckstrin Homology
PI	Phosphoinositide
PI_3K	Phosphoinositide 3 Kinase
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein Phosphatase 2A
SAPK/JNK	Stress Activated Protein Kinase/ c-Jun N-terminal Kinase
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
TBS	Tris Buffered Saline
TCA	Trichloroacetic Acid
TLC	Thin Layer Chromatography
ζ	Zeta

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Chapter 1

LITERATURE REVIEW

Experimental approaches described in this thesis focus on certain mechanisms by which mitogenesis is controlled during the inflammatory process. Specifically, experiments are described to define the roles that lipid-derived second messengers play in limiting cellular proliferation despite inflammation. These lipid second messengers may provide therapeutic opportunities in such chronic inflammatory conditions as atherosclerosis and restenosis. Our studies are designed to target signal transduction cascades that are activated by second messengers to limit proliferation during inflammation.

Cellular homeostasis is maintained through the fine balance between mitogenesis and apoptosis. The mechanisms by which cells maintain this homeostasis are highly active areas of research. An underlying theme in these areas of research is that regulation of homeostasis is exceedingly complex, involving many different molecules and pathways. In disease states, this balance is often skewed either toward proliferation or cell death. One benefit of determining how cellular homeostasis is maintained is the ability to manipulate the balance favorably in disease states.

Chronic inflammation is problematic since it is usually associated with irreversible damage or destruction of normal parenchyma, followed by replacement with scar tissue. Furthermore, the chronic inflammatory response is a common denominator in various disease processes. Therefore, the ability to

control or limit the regulation of this response is desirable as a potential target for drug therapy in the prevention or control of human disease. Atherosclerosis and restenosis are examples of chronic inflammatory diseases. Chronic inflammation is activated by chemokines and cytokines that maintain or augment the inflammatory response. In addition, growth factors are released from activated cells. However, the mechanisms to limit growth factor-induced proliferation during inflammation have not been adequately explored.

During inflammation, specific stimuli selectively activate many intracellular signal transduction cascades. Traditionally, signal transduction cascades have been considered to be linear, where one kinase phosphorylates and activates an immediate downstream kinase, and so forth. However, we believe that the activated cascades are regulated by the formation of signaling kinase complexes. Therefore, we investigated the ability of lipid second messengers to regulate formation of these signaling complexes, and hence, cascade activation. Furthermore, we hope to modulate selectively the proliferative signals by using ceramide, a growth-arresting lipid.

Two signaling pathways in mammalian cells are important for determining the cellular phenotype: the ERK (Extracellular Signal-Regulated Kinase) and SAPK pathway (Stress Activated Protein Kinase Pathway, also known as JNK, c-Jun N-terminal Kinase). Both the ERK and SAPK pathways are members of the Mitogen Activated Protein Kinase (MAPK) cascade family. Another relevant pathway is the PI_3K (Phosphoinositide 3 Kinase)/Akt1 pathway. These pathways are activated by specific stimuli. The ERK cascade is associated with growth

factor-induced proliferation, whereas the SAPK pathway is activated by stress, e.g. changes in osmolarity and cytokines, such as IL-1. We are interested in defining and exploiting therapeutically the differences between these two MAPK cascade family members. Our hypothesis is that ceramide can inhibit the ERK cascade while activating the SAPK cascade, thereby resulting in overall cell growth arrest. The PI_3K pathway, also activated by growth factors, such as PDGF and IGF, is a key pathway for cell survival. Therefore, we are also interested in investigating the ability of ceramide to modulate this pathway. This thesis explores the ability of ceramide, a sphingomyelin metabolite, to regulate protein kinase cascades and reduce inflammation in activated vascular smooth muscle cells (**Figure 1**).

1.1 Sphingomyelin

Activation of endogenous signal transduction cascades, such as ERK, SAPK and PI_3K, begins at the plasma membrane where growth factors and cytokines bind to their cellular receptors. The plasma membrane is composed primarily of phospholipids, cholesterol and sphingolipids. These lipids serve as the structural basis for the plasma membrane. However, in recent years, it has become apparent that the plasma membrane does more than act as a structural scaffold for the cell. Microdomains of lipids in the plasma membrane rich in sphingomyelin and cholesterol, called rafts or caveolae, serve as highly concentrated areas for many signaling molecules that are lipid cofactors and/or activators, such as ceramide and diacylglycerol [Shaul & Anderson, 1998; Brown

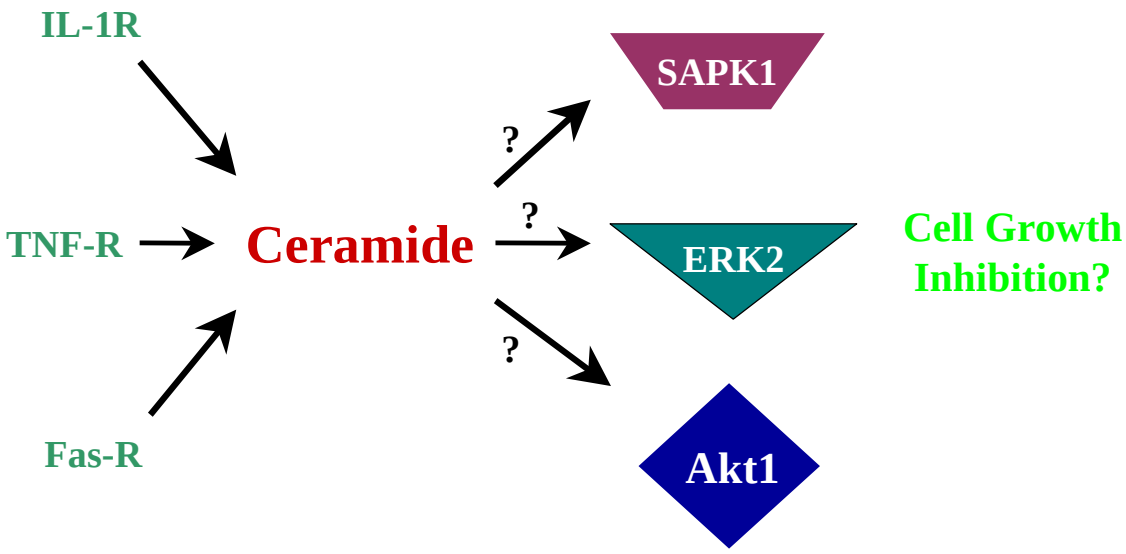


Figure 1. Proposed ceramide regulation of select signal transduction cascades.

& London, 1998; Dobrowsky, 2000; Dobrowsky & Gazula, 2000]. These specialized domains may serve as signaling foci for signal transduction cascades.

The predominant sphingolipid identified in plasma membranes, as well as in membrane rafts or caveolae, is sphingomyelin. Analogous to phospholipids, sphingomyelin generates bioactive second messengers, such as ceramide and sphingosine. The sphingomyelin metabolic pathway, shown in **Figure 2**, is activated during biological, chemical or physical stresses that include TNFα, chemotherapeutic drugs, changes in osmolarity, and UV light, [Hannun & Obeid, 1997]. Such stresses to stimulate signaling pathways that lead to growth arrest, differentiation or apoptosis.

Once ceramide is generated from sphingomyelin, it can then serve as a cofactor for cellular targets before being either reincorporated into the plasma membrane or further metabolized. Alternatively, growth factors can activate the enzyme ceramidase, leading to the ceramide catabolism and generation of sphingosine. The net result of sphingosine generation is the activation of signaling cascades that lead to proliferation rather than growth arrest. The importance of the sphingomyelin pathway in response to stress is demonstrated by the fact that it is evolutionarily conserved from yeast to mammals, thus demonstrating the fundamental importance of this pathway. In fact, studies in yeast suggest that this pathway may actually predate apoptosis as a stress response [Dickson *et al*., 1997; Jenkins *et al*., 1997; Mandala *et al*., 1998].

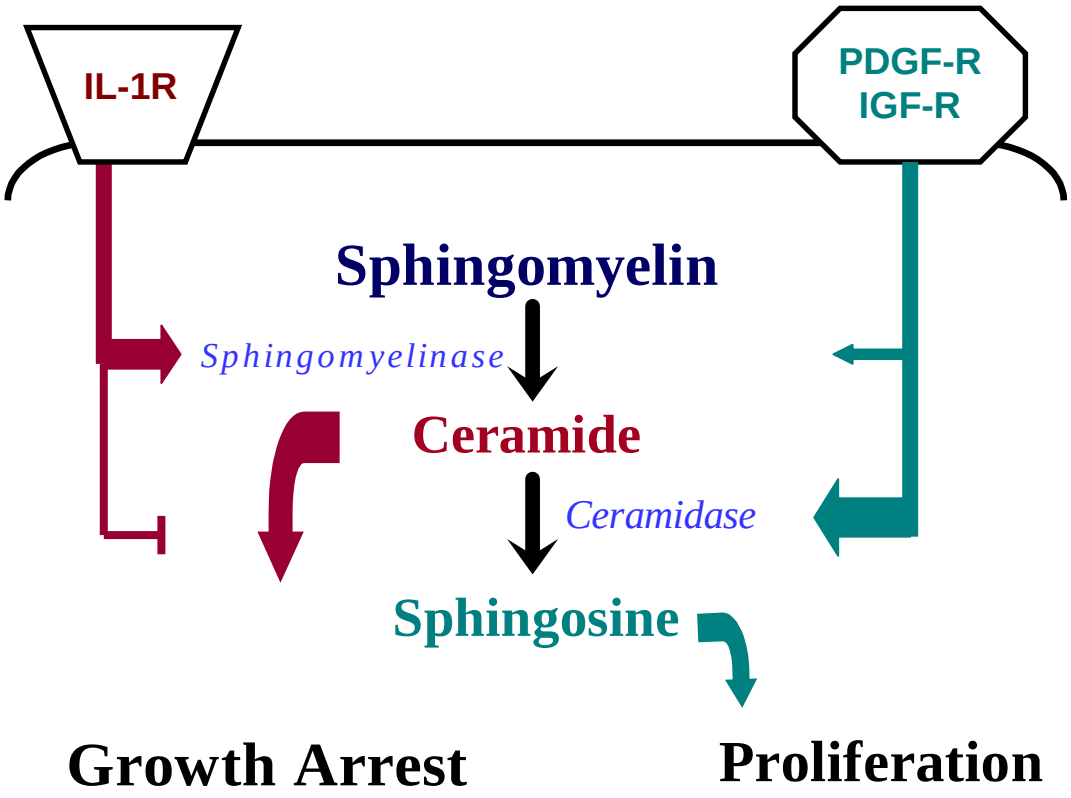


Figure 2. The Sphingomyelin Metabolic Pathway

In addition to the catabolic production of ceramide, synthesis of new ceramide can be generated *de novo*, as shown in **Figure 3**. Ceramide generated *de novo*, as a result of such stimuli as TNFα and chemotherapeutic drugs, results in apoptosis [Bose *et al*., 1995; Garzotto *et al*., 1998; Paumen *et al*., 1997]. However, the effectors by which apoptosis is induced are thought to differ from those used by sphingomyelin-generated ceramide, as there are differences in caspase activation between the two species of ceramide [Perry & Hannun, 1998]. Ceramide can also be post-translationally modified into glycosphingolipids or may be phosphorylated. The glycosphingolipids, generated from the precursor glucosylceramide, have been linked to regulation of apoptosis and tumor metastasis [De Maria *et al*., 1997; Inokuchi *et al*., 1990]. Ceramide-1-phosphate, generated by phosphorylation of ceramide via ceramide kinase, has been associated with proliferation; however, little is presently known about this lipid species [Gomez-Munoz *et al*., 1995].

1.2 Ceramide

Our studies focus on the sphingomyelin metabolite ceramide. Inflammatory cytokines, including interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ), activate sphingomyelinases, which subsequently hydrolyze sphingomyelin, resulting in increased cellular ceramide concentrations [Coroneos *et al*., 1995; Kim *et al*., 1991; Mathias *et al*., 1993]. Ceramide has been implicated in growth inhibition, apoptosis, cell senescence and cellular differentiation [Jarvis *et al*., 1994; Jayadev *et al*., 1994]. The actions

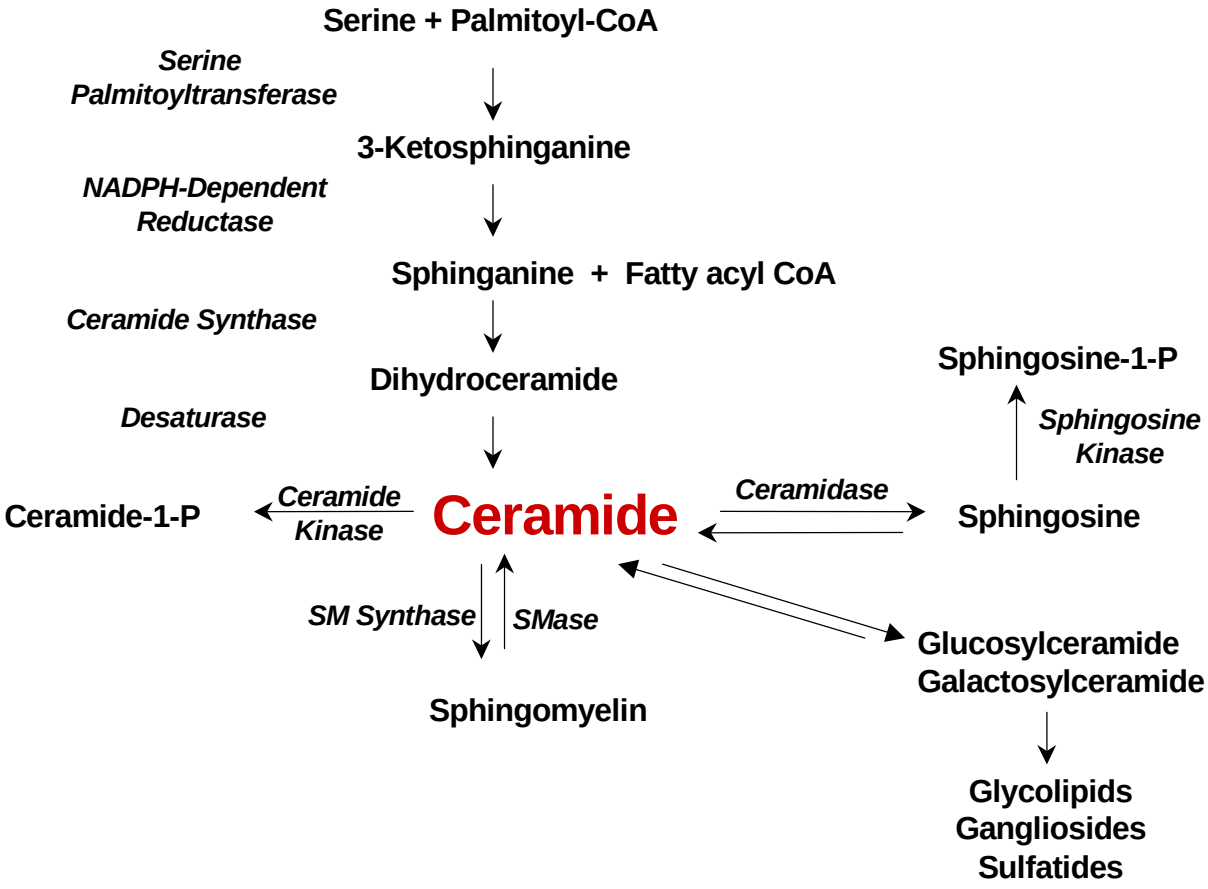


Figure 3. The biosynthetic pathway for ceramide synthesis.

of ceramide are both stereospecific and structurally selective. The physiological C_{18}-ceramide has a D-*erythro* conformation. Other stereoisomers of ceramide lack biological activity. In addition, there is a critical double bond at the 4,5 position. Without this double bond, ceramide has no biological activity. In fact, this lipid species, called dihydroceramide, is used as a negative control in many experiments. Our laboratory previously demonstrated that the cell-permeable ceramide analogue, C_6-ceramide, mimics the effect of IL-1 to inhibit both tyrosine kinase receptor- and G-protein receptor-linked mitogenesis [Coroneos *et al*., 1996; Mandal *et al*., 1997]. Mechanisms by which ceramide induce cell cycle arrest currently remain undefined.

Ceramide can exert its growth arresting, differentiating or apoptotic effects by various mechanisms. Several direct targets of ceramide have been identified, including ceramide-activated-protein kinase (CAPK) [Joseph *et al*., 1993; Mathias *et al*., 1991], ceramide-activated protein phosphatase (CAPP) [Dobrowsky & Hannun, 1993; Dobrowsky *et al*., 1993; Fishbein *et al*., 1993], caspases [Kojima & Datta, 1996; Mizushima *et al*., 1996; Smyth *et al*., 1996] and Protein Kinase C zeta (PKCζ) [Lozano *et al*., 1994; Muller *et al*., 1995]. CAPK is a proline directed serine/threonine protein kinase reported to be a Kinase Suppressor of Ras (KSR) [Zhang *et al*., 1997]. One laboratory has demonstrated that ceramide may induce apoptosis through KSR, and hence Ras and Raf-1, via the presence of Bad, a member of the Bcl-2 family [Basu *et al*., 1998]. However, the role of this putative ceramide target remains a subject of much controversy in the literature. CAPP has been identified as a member of the protein phosphatase 2A family

[Dobrowsky & Hannun, 1993; Dobrowsky *et al*., 1993]. CAPP may mediate the growth arresting effects of ceramide by dephosphorylating the retinoblastoma gene product, thus resulting in cessation of cell cycle progression at Go/G1 [Alberts *et al*., 1993]. These targets of ceramide, as well as other unidentified or indirect targets, may interact with various proteins and kinases to influence the cellular fate.

There are several possible mechanisms by which ceramide may use these and other targets to execute its effects. The most obvious mechanism is by ceramide-induced phosphorylation or de-phosphorylation of key signaling proteins. These events may occur indirectly via CAPP and CAPK. Ceramide may also induce the cleavage of other proteins, such as caspases, thus generating additional bioactive proteins that may serve as effectors to induce apoptosis. Another possibility is that ceramide may bind to and inhibit or sterically hinder other proteins so that they cannot be activated by their various cofactors or kinases. Whatever the mechanism, it seems likely to involve extensive cross talk of multiple signaling cascades.

1.3 Protein Kinase C

The known ceramide target focused on in these experiments is PKCζ. PKCζ is a member of the PKC family of kinases, discovered in the late 1970s [Inoue *et al*., 1977; Kishimoto *et al*., 1977; Takai *et al*., 1977]. PKCs are serine/threonine kinases activated by phosphatidylserine. There are at least 11 PKC isoforms, subdivided into 3 general categories: conventional (α, βI, βII, γ),

novel (δ, ε, θ, η, μ) and atypical (ζ, λ/ι). PKCs are classified based upon phospholipid-derived cofactors, such as DAG, Ca^{2+} and phorbol esters. The conventional class of PKCs is Ca^{2+}-dependent and DAG-dependent. The novel class is DAG-dependent, but Ca^{2+}-independent. The atypical class is both Ca^{2+}- and DAG-independent. Cofactor dependency for PKCs is dictated by differences in domain localization or by omission from the protein. For example, the Ca^{2+}-binding domain is thought to be located in the C2 domain. The conventional class of PKCs not only has the C2 domain, but is also located in the proper position in the primary structure. The novel class, although possessing a C2 domain, does not require Ca^{2+} for activation, as the C2 domain is not in the proper location.

The primary structures for the different classes of PKCs are shown in **Figure 4**. In addition to differences in co-factor dependency, PKCs exhibit differences in tissue and cellular localization [Dekker & Parker, 1994; Hug & Sarre, 1993; Newton, 1995; Newton, 1997]. This family of kinases phosphorylates other proteins with the basic consensus sequence RXXS/TXRX, where X represents any amino acid. However, subtle differences in this consensus sequence exist among the PKC family members [Nishikawa *et al*., 1997]. PKCs have been linked to a variety of cellular processes, including remodeling of the actin cytoskeleton, modulation of ion channels, mitogenesis, apoptosis, regulation of select transcription factors and secretion, as well as many others [Toker, 1998].

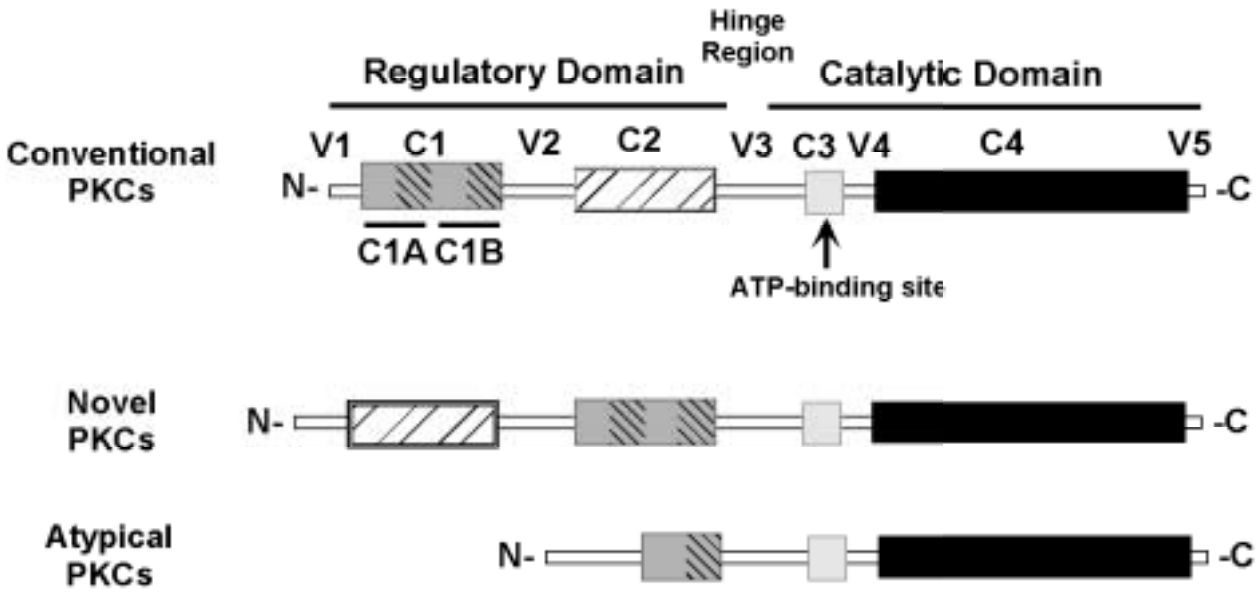


Figure 4. Schematic for the primary structure of PKCs.

Our studies focus on members of the novel and atypical PKC classes, as these two classes are both Ca^{2+}-independent. We are interested in Ca^{2+}-independent classes since IL-1, which endogenously generates ceramide, does not increase significantly intracellular calcium levels. Specifically, experiments assess the regulation of PKCε and PKCζ, members of the novel and atypical classes of PKCs, respectively, by ceramide. These two isoforms are associated with phenotypic differences. PKCζ has been associated with a growth-arrested phenotype [Montaner *et al*., 1995; Kieser *et al*., 1996]. Conversely, PKCε has been associated with proliferation [Toker, 1998; Sasaguri *et al*., 1993; Cacace *et al*., 1993]. Although, there are other PKC isoforms that are important for the cellular phenotype, experiments described in this thesis are focused on PKCζ and PKCε, as these are predominant isoforms in our cellular model. However, it is possible that mechanisms of regulation by ceramide described in this thesis may apply to other members of the PKC superfamily.

1.4 Stimulatory Actions of Ceramide

A. PKCζ

PKCζ, a putative ceramide-activated protein kinase involved in growth control, is directly activated by ceramide and not by diacylglycerol (DAG) [Lozano *et al*., 1994; Muller *et al*., 1995; Galve-Roperh *et al*., 1997; Wang *et al*., 1999]. In addition, ceramide-induced growth arrest has been related to activation of SAPK/JNK, but the precise mechanism remains to be elucidated [Coroneos *et al*., 1996; Westwick *et al*.; 1995; Verheij *et al*., 1996]. As ceramide activates

PKCζ and SAPK in the same cell type, it has been inferred, but not proven, that PKCζ is a critical element in ceramide-induced SAPK activation [Wang *et al*., 1999]. It seems likely that the site of action for ceramide is upstream of SAPK, since ceramide does not directly regulate immunoprecipitated SAPK activity in a cell-free system [Mandal *et al*., 1997]. One hypothesis of this thesis is that ceramide induces growth arrest through ceramide-activated PKCζ recruitment of upstream components of the SAPK pathway into a signaling complex, resulting in activation of the SAPK cascade.

B. SAPK

The SAPK cascade is linked to activation by a variety of stress stimuli, such as cytokine stimulation or changes in osmolarity. Activation of the SAPK signaling pathway is characterized by a cascade of protein kinases recruited to the plasma membrane. Specifically, GTP-dependent activation of the low molecular weight G-proteins Rac and Cdc42 leads to recruitment of MEKK1 to the plasma membrane, where it is phosphorylated and activated [Coso *et al*., 1995]. Stimulated MEKK1 directly phosphorylates and activates SEK, which in turn directly phosphorylates SAPK. SAPK then can phosphorylate c–Jun, resulting in activation of this AP-1 transcription factor [Whitmarsh & Davis, 1996; Whitmarsh *et al*., 1995]. Stimulation of Rac-1 by inflammatory cytokines or ceramides has been postulated to be one mechanism to activate the SAPK cascade [Coso *et al*., 1995; Brenner *et al*., 1997; Shin *et al*., 1999]. As PKCζ does not directly regulate Rac-1 [Uberall *et al*., 1999], an alternative mechanism

for stimulation of SAPK could be ceramide-induced PKCζ activation of MEKK1 and/or SEK. Thus, it is hypothesized that ceramide regulation of the SAPK pathway may also be dependent upon direct activation of PKCζ.

In our studies, we demonstrate that ceramide directly up-regulates both immunoprecipitated and recombinant human PKCζ activity. Upon ceramide treatment, PKCζ interacts with MEKK1, SEK and SAPK to inhibit Insulin-like Growth Factor-1 (IGF-1)-induced cell proliferation. Together, these findings suggest a novel role for ceramide in regulating and assembling multi-protein signaling complexes.

1.5 Inhibitory Actions of Ceramide

A. ERK and PKCε

Inhibitory action of ceramide in cell growth involves inhibition of ERK activity, a member of the MAPK family [Coroneos *et al*., 1996; Muller *et al*., 1998]. To date, however, the precise role of ceramide in inhibition of ERK activation, as well as cell growth, has not been determined. It is likely that the active site of ceramide is upstream of ERK since ceramide does not directly regulate immunoprecipitated ERK activity in cell-free systems.

Activation of the ERK signaling pathway by growth factors is characterized by a cascade of protein kinases recruited to the plasma membrane. Specifically, GTP-dependent activation of Ras recruits Raf-1 to the plasma membrane, where it is phosphorylated and activated [Yao *et al*., 1995]. Stimulated Raf-1 directly phosphorylates and activates MEK, which in turn directly phosphorylates ERK.

Upon ERK phosphorylation, it can translocate to the nucleus, where it phosphorylates ternary complex transcription factors [Whitmarsh & Davis, 1996]. ERK may also phosphorylate other proteins, such as histone or myelin basic protein, which can be used as exogenous phosphorylation substrates for *in vitro* experiments [Lenormand *et al*., 1993]. In addition to Ras, PKCε also activates the Raf-1-MEK-ERK signaling pathway [Cacace *et al*., 1996].

In response to growth factors such as insulin-like growth factor-1 and platelet-derived growth factor (PDGF), PKC is activated through phospholipase C-generated diacylglycerol (DAG). Among the PKCs, DAG-regulated PKCε, a member of the novel class of PKC isotypes, activates Raf-1 kinase [Sozeri *et al*., 1992; Ueffing *et al*., 1997]. Over-expression of active PKCε overcame the inhibitory effects of dominant negative Ras, suggesting that PKCε-induced activation of the Raf-1-MEK-ERK signaling cascade is independent of Ras activation [Cacace *et al*., 1993; Cacace *et al*., 1996; van Dijk *et al*., 1997]. We previously reported that the activity of PKCε is significantly inhibited by IL-1 treatment in rat mesangial cells [Mandal *et al*., 1997]. Furthermore, we demonstrated that the cell-permeable ceramide analogue, C_6-ceramide, mimicked the effect of IL-1 to inhibit both tyrosine kinase receptor- and G-protein receptor-linked mitogenesis [Coroneos *et al*., 1996; Mandal *et al*., 1997]. As ceramide is structurally similar to DAG, the endogenous co-factor for PKCε activation, inhibitory action of ceramide upon growth factor-induced ERK activation and subsequent cell growth inhibition may be due to the antagonistic action of ceramide displacing DAG on PKCε.

As PKCε has been shown to phosphorylate Raf-1, one upstream component of ERK, we hypothesize that ceramide inactivates PKCε, resulting in decreased complex formation with upstream components of the ERK cascade. The inability of PKCε to interact with upstream components of the ERK cascade should lead to decreased activation of the ERK pathway. If correct, this hypothesis would define a novel role for ceramide in modulating the physical interactions between signaling elements in PKC•MAPK complexes, thus inactivating the proliferative ERK cascade.

B. Akt-1 and PKCζ

In addition to stimulating the ERK cascade, growth factors as well as survival factors induce activation of PI_3K [Toker & Cantley, 1997]. PI_3K stimulation has been associated with a variety of cellular responses, such as gluconeogenesis, cell survival and transformation [Toker & Cantley, 1997]. PI_3K phosphorylates phosphoinositides at the 3-position of the inositol ring in inositol phospholipids, which subsequently interact with proteins containing pleckstrin homology (PH) domains [Falasca *et al*., 1998; Hemmings, 1997a; Hemmings, 1997b]. Pleckstrin homology domains are regions that are thought to be important for membrane targeting and, in some cases, interaction with other proteins. One downstream kinase with a PH domain that is a key mediator of PI_3K is the serine/threonine kinase Akt-1 (also known as protein kinase B).

There are three known isoforms of Akt : Akt-1, Akt-2 and Akt-3. The best characterized of these isoforms is Akt-1. Akt-1 exists primarily as a cytoplasmic

protein; however, it has also been identified in the nucleus [Andjelkovic *et al*., 1997; Meier *et al*., 1997]. Akt-1 requires several phosphorylation sites for optimal kinase activity. There are two obligatory phosphorylation sites necessary for Akt activation, threonine 308 and serine 473 [Alessi *et al*., 1996; Bellacosa *et al*., 1998]. In addition to these 2 critical phosphorylation sites are two other important phosphorylation sites, serine 124 and threonine 450. Ser124 and Thr450 are considered to be constitutively phosphorylated. In addition, Thr450 is thought to be indicative of proper protein folding [Bellacosa *et al*., 1998]. All of these phosphorylation sites are potential sites for inhibition of Akt-1.

Phosphorylation sites are not the only potential sites of inhibition on Akt-1. Akt-1 contains a pleckstrin homology (PH) domain that is required for membrane targeting. In addition, the PH domain associates with PH-interacting proteins, such as phosphoinositide(3,4,5)P_3-dependent 3-kinase 1 (PDK1) and PKCζ. Therefore, binding to the PH site may serve as a means to inhibit Akt-1 membrane localization and/or interaction with activating lipids or proteins. Upon recruitment to the membrane, Akt-1 is phosphorylated at Thr308 and Ser473 and fully activated. Phospho-Akt-1 is then able to translocate to the nucleus where it is able to regulate downstream “proliferative” proteins or stimulate protein synthesis, such as cyclin D and the transcription factor E2F [Andjelkovic *et al*., 1997; Brennan *et al*., 1997; Meier *et al*., 1997].

Several studies have demonstrated that ceramide inhibits Akt-1 [Salinas *et al*., 2000; Schubert *et al*., 2000; Summers *et al*., 1998; Zhou *et al*., 1998]. However, the precise mechanism by which this occurs is unknown. As studies

have demonstrated PKCζ directly binding to Akt-1 [Konishi *et al*., 1994a; Konishi *et al*., 1994b], we hypothesize that ceramide-mediated inhibition of Akt-1 depends upon PKCζ. Supporting this hypothesis are recent studies showing that PKCζ inactivates Akt-1 via phosphorylation [Konishi *et al*., 1994a; Konishi *et al*., 1994b]. As mentioned above, Akt-1 is a well-established downstream effector of PI_3K. Whether ceramide inhibits Akt-1 through a PI_3K-dependent mechanism is still a subject of controversy in the literature. Reports claim that ceramide inhibition is PI_3K-dependent [Zundel & Giaccia, 1998] and PI_3K-independent [Chen *et al*., 1999; Meier *et al*., 1998]. In either case, PKCζ may represent a novel component for mediating ceramide-induced inhibition of Akt-1.

Chapter 2

MATERIALS AND METHODS

Human Embryonic Kidney 293 (HEK 293) cells were obtained from American Type Culture Collection (Rockland, MD). Anti-PKCα, -PKCε -PKCζ, -PCNA, Raf-1 -ERK, -pERK, -SAPK, -SEK1/MEK4 and –MEKK1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody for the phosphorylated SEK was obtained from New England Biolabs (Cambridge, MA). Purified, recombinant PKCζ and PKCε were obtained from Panvera Corp. (Madison, WI). Both cell-permeable C_6-ceramide and physiological $C_{18:1}$-ceramide were obtained from Avanti Polar Lipids (Alabaster, AL). In addition, the inactive cell-permeable ceramide analogue, dihydro-C_6-ceramide (DH), was obtained from Biomol (Plymouth Meeting, PA). Diacylglycerol (1,2-Diolein, DAG) and 1-Oleoyl-2-Acetyl-*sn*-Glycerol (OAG) were purchased from Serdary Research Laboratories (Ontario, Canada). Human IGF-1, PDGF-ββ and IL-1β recombinant proteins were purchased from GIBCO (Grand Island, NY). The ECL detection kit was obtained from Amersham Life Science (Arlington Heights, IL).

2.1 CELL CULTURE

Human Embryonic Kidney (HEK 293) and A7r5 cells

HEK 293 cells are adenovirus-transformed human embryonic kidney cells of tubule epithelial origin. HEK cells were maintained in DMEM supplemented with 10% FBS at 37°C with 5% CO_2.

For select experiments, specifically for Akt-1/ PKCζ experiments, we utilized A7r5 cells, a rat thoracic aortic smooth muscle cell line. A7r5 cells were maintained in DMEM supplemented with 10% FBS and antibiotics at 37°C with 5% CO_2.

2.2 WESTERN ANALYSES

HEK 293 and A7r5 cells: Tissue Preparation and Western Blot Analyses

Western blot analyses using anti-PKCζ or anti-PKCε antibodies were performed as previously described [Mandal *et al*., 1997]. Briefly, HEK 293 cells were grown in either 6-well plates or 60-mm dishes, down-regulated and treated. Cells were then washed in ice-cold Dulbecco's PBS solution and lysed in 0.3-0.4 ml of lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1mM EGTA, 1 mM $NaVO_4$, 0.2% NP-40, 1 μg/ml each of leupeptin, pepstatin, and aprotinin). Cell lysates were cleared by centrifugation, and the protein concentration was determined via the Bio-Rad protein assay. Forty micrograms of protein lysate per sample was separated on a 12% SDS-PAGE gel and transferred to Hybond nitrocellulose membranes. The membranes were blocked in 5% nonfat milk in tris-buffered saline (TBS) for 2 hours and then incubated with the primary anti-PKCζ or –PKCε antibody (1:1000 dilution in 5% nonfat milk TBS) for 2 hours at room temperature. After incubation, the membranes were washed three times with TBS for 10 minutes each. The blots were then incubated with secondary HRP-conjugated goat anti-rabbit IgG antibody (1:5000 dilution in 5% nonfat milk in TBS) for 1-2 hours at room temperature. The membranes were

then washed three times with TBS. The PKCζ or PKCε band was visualized by ECL and quantified using laser densitometry. To confirm the specificity of the antibodies utilized for immunoprecipitation of PKCε and PKCζ, we reprobed the membranes with additional PKC isotype-specific antibodies. Bands corresponding to the molecular weights of PKCε or -ζ were observed when the membranes were probed with the antibodies for PKCε or -ζ, respectively. In contrast, bands were not observed when the membranes were probed with antibodies for α, δ or ζ (data not shown). A similar protocol was used for detection of ERK, phosphorylated ERK (pERK), Raf-1, PCNA, MEKK1, p-SEK, SAPK, Akt1 and p-Akt1 expression.

2.3 KINASE ASSAYS

A. HEK 293 cells: *In Vitro* Reconstitution Assay for Immunoprecipitated and Recombinant PKCs

Immunoprecipitation of PKCζ and -ε and the subsequent reconstitution activity assays were adapted from previous methods [Biswas *et al*., 1995; Mandal *et al*., 1997; Musial *et al*., 1995]. HEK 293 cell were grown in 6-well or 60 mm dishes, down-regulated for 24 hours and then lysates prepared (as described above). PKC isoforms were immunoprecipitated from HEK 293 lysates using 0.5 μg of polyclonal rabbit anti-PKC antibody. After overnight incubation at 4°C, goat anti-rabbit IgG agarose was added, rotated for 2 hours at 4°C, and the immunocomplexes containing specific PKC isoforms were pelleted by brief low-speed centrifugation. After two washes with PBS and one wash with

kinase buffer, the pellets were resuspended in kinase buffer (50 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, 50 mM NaF, 1 mM $NaVO_4$, 1 mM DTT, 0.1% Tween 20). The *in vitro* kinase reaction was initiated by addition of 40 μg/ml phosphatidylserine/reaction, 10 mM $MgCl_2$, 0.25 mM ATP and 1 μCi [γ-^{32}P] ATP (10 mCi/mmol) and 10 μg histone IIIS as a substrate. Specified samples contained DAG (1,2 diolein, 1 μM) and/or C_6-ceramide, dihydro-C_6-ceramide, $C_{18:1}$-ceramide (0.1-1μM) as co-factors. After a 15 minute incubation with shaking at 37°C, the kinase reaction was terminated by addition of sample buffer and heated at 95°C for 5 minutes. Phosphorylated histone IIIS proteins were then separated on 12% SDS-PAGE gels and transferred to Hybond nitrocellulose membranes. The bands corresponding to phosphorylated histone IIIS were detected by autoradiography (Kodak X-OMAT). In other experiments, samples were transfected with HA-tagged PKCε, immunoprecipitated with anti-HA antibody, and then a kinase assay was performed as previously described.

In selected experiments, 10μg of myelin basic protein was used as the exogenous substrate, rather than histone IIIS. The bands corresponding to phosphorylated myelin basic protein were excised and quantified by liquid scintillation analysis. A similar protocol utilizing recombinant PKCζ or -ε (50 ng) was used to verify the *in vitro* effects of physiological DAG and ceramide upon immunoprecipitated PKC isoforms.

B. A7r5 cells: *In Vitro* Kinase Assay for Immunoprecipitated PKCζ

Immunoprecipitation of PKCζ from A7r5 was performed with a protocol as described above. A7r5 cells were grown in 60 mm dishes until about 90% confluent. Cells were then down-regulated with unsupplemented DMEM for 24 hours. Select cells were pre-treated with LY294002 (1 μM) or Wortmannin (100 nM) for 30 minutes and subsequently treated either with C_6-ceramide or DH-ceramide for 1 hour or PDGF (10 ng/ml) or IL-1 (20 ng/ml) for 5 minutes. Lysates were prepared as previously described and protein levels determined by the Bio-Rad assay. Lysates were immunoprecipitated with anti-PKCζ antibody for 3 hours at 4°C. Immunocomplexes were captured with gamma-bind sepharose for 2 hours at 4°C. Pellets were washed twice with PBS and once with kinase buffer (previously described). A kinase assay was then performed using 10 μg of myelin basic protein as a substrate, in the presence of 40 μg/ml phosphatidylserine and 1 μCi [γ-^{32}P] ATP (10 mCi/mmol), for 15 minutes. The kinase reaction was terminated by the addition of SDS sample buffer and then heated for 5 minutes at 95°C. Samples were separated on 12% SDS-PAGE gels and then transferred to nitrocellulose membranes. Phosphorylation of MBP was assessed by liquid scintillation counting of the excised MBP band.

C. HEK 293 cells: *In Vitro* Reconstitution Assay for Immunoprecipitated SAPK

In vitro SAPK kinase assays were performed as previously described [Coroneos *et al*., 1996]. HEK 293 cells were transfected with either wild-type

PKCζ or dominant-negative PKCζ. Cells were allowed to recover, down-regulated in low-serum (0.05% FBS) DMEM for 24 hours, treated with either vehicle (0.01% DMSO) or (1 μM) C_6-ceramide and then lysed with previously described lysis buffer. Briefly, 400 μg of protein was immunoprecipitated with anti-SAPK1 antibody overnight at 4°C. Immunoprecipitates were then captured with gamma-bind sepharose for 2 hours at 4°C. Immunocomplexes were pelleted and resuspended in kinase buffer. The reaction was initiated by the addition of 20 μM ATP (10 μCi ATP/assay) and c-jun, as the exogenous phosphorylation substrate. The phosphorylated c-jun bands were detected by autoradiography.

D. A7r5 cells: PI_3K assays

A7r5 cells were grown to confluence in 100 mm dishes and down-regulated in DMEM (containing 0% FBS) for 24 hours. Whole cell experiments included dishes of cells pre-treated with C_6-ceramide for 2 hours. Select dishes were subsequently treated with or without PDGF (10 ng/ml) for 5 minutes and then lysates prepared. Cell lysates were cleared by centrifugation, and the protein concentration was determined via the Bio-Rad protein assay centrifuged at 14,000 RPM. Lysates (400 μg) were then immunoprecipitated with anti-p85α antibody overnight at 4°C. Immunocomplexes were captured by a 2-hour incubation with gamma-bind sepharose. Pellets were then washed once with lysis buffer and twice with kinase buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 10 mM $MgCl_2$). Cell-free samples were pre-treated with ceramide

for 10 minutes. A kinase assay was then performed on both whole cell and cell-free samples for 15 minutes at 35°C, in the presence of 20 μg/ml phosphatidylserine and 20 μM ^{32}P-ATP (10 μCi ATP/assay), using 200 μg/ml phosphoinositide (PI) as the exogenous substrate. The kinase reaction was terminated by the addition of 200 μl of 1 M HCl-Methanol (1:1). Lipids were extracted twice with 200 μl of chloroform and then dried under nitrogen. Forty microliters of chloroform was then added to each assay tube and then spotted onto a TLC plate (Whatman Silica 60, aluminum). The lipids were separated on the TLC plate with a solvent system consisting of 60:47:11.3:2 Chloroform: Methanol: Water: Ammonium Hydroxide. After the samples separated, the TLC plates were removed, air-dried and placed in a –80°C freezer for an appropriate length of time. Phosphorylation of PI substrate was assessed by liquid scintillation counting.

2.4 CO-IMMUNOPRECIPITATION EXPERIMENTS

A. HEK 293 cells: Co-immunoprecipitation of PKC with MAPK cascade Signaling Components

Lysates from acutely treated HEK 293 cells were prepared as previously described and incubated with rabbit anti-SAPK1, anti-PKCζ, anti-MEKK1, anti-PKCε, anti-ERK, or anti-Raf-1 antibody for 12 hrs at 4°C. Goat anti-rabbit IgG antibody conjugated to agarose or gamma-bind sepharose was added to each sample and incubated for 2 hours at 4°C. Immunocomplexes were then pelleted by brief low speed centrifugation and washed twice in lysis buffer and washed

once in kinase buffer. Immunoprecipitates were combined with sample buffer and heated at 95^{o}C for 5 minutes, followed by separation on 12% SDS-PAGE gels. Proteins were transferred to Hybond nitrocellulose membranes and probed with anti-PKCζ anti-pSEK, anti-PKCε or anti-pERK antibody (1:1000 dilution in 5% nonfat milk in TBS). Subsequently, the membranes were incubated with the HRP-conjugated anti-rabbit IgG antibody (1:5000) and the bands corresponding to PKCζ, pSEK, pERK or PKCε were visualized by ECL. Equal loading of SAPK, MEKK1, PKCζ, PKCε, ERK2 or Raf-1 was determined by reprobing the membranes with anti-SAPK, -MEKK1, -PKCζ, -PKCε, -ERK2 or –Raf-1 antibodies.

B. A7r5 cells: Co-immunoprecipitation of PKCζ with Akt-1

A7r5 cells were grown to 75% confluence in 60 mm dishes. Cells were then down-regulated in non-supplemented (0% FBS) DMEM for 24 hours. Cells were pretreated for 1 hour with or without C_6-ceramide (1 μM) or biologically inactive DH-C_6-ceramide (1 μM) and then treated with IGF-1 (50 ng/ml) for 5 minutes. Lysates were prepared as previously described. A7r5 lysates were then immunoprecipitated with anti-Akt1 antibody and then assessed for co-immunoprecipitation with PKCζ or PKCε by Western analysis. PKC bands were visualized by ECL®.

2.5 TRANSIENT TRANSFECTIONS

A. Transfection of HEK 293 cells with PKCζ constructs

HEK 293 cells were transiently transfected using Superfect® (Qiagen) according to manufacturer instructions. HEK 293 cells were transfected with either wild-type or dominant-negative mutant PKCζ constructs (a generous gift from Dr. J. Moscat). The wild-type PKCζ construct is a full length PKCζ in a pCDNA3 expression vector. The dominant-negative mutant PKCζ construct is a kinase defective mutant that contains a point mutation in the catalytic domain. The constitutively-active PKCζ construct is the catalytic domain without the regulatory domain. After transfection for 24 hours, cells were washed once with PBS and down-regulated for 24 hours in DMEM (0% FBS). Cells were then treated with either C6-ceramide or vehicle (0.01% DMSO) for 10 minutes. Lysates were prepared as described above. Cells expressing the wild-type and dominant-negative mutant constructs were subjected to *in vitro* kinase assays to assess SAPK bioactivity, as described above, using c-jun as the exogenous phosphorylation substrate.

In other experiments, HEK cells were transfected with wild-type or constitutively-active PKCζ. After transfection, as described above, cells were pretreated with C_6-ceramide or DH-C_6-ceramide for 1 hour or IL-1 (20 ng/ml), followed by treatment with OAG (1 μM) or IGF-1 (50 ng/ml) for 18-20 hours. Lysates were obtained and subjected to Western blot analysis to assess Proliferating Cell Nuclear Antigen (PCNA) expression, a general marker of cells undergoing the cell cycle. To verify that the transfections with PKCζ constructs

did not alter protein levels of other PKC isoforms, Western analyses were performed, and PKCα and PKCε expression did not change (data not shown).

B. Transfection of A7r5 cells with PKCζ constructs

A7r5 cells were transfected with transiently transfected with empty vector, wild-type or dominant-negative mutant PKCζ constructs (a generous gift from Dr. J. Moscat) using Superfect® (Qiagen). The same protocol for PKCζ constructs was followed as described above. Transfection efficiency was determined to be ~25-30%, as assessed by green fluorescent protein co-transfection. Cells were then pre-treated with either C_2- or C_6-ceramide (1 μM) for 1 hour and then treated with PDGF (10 ng/ml) for 5 minutes. Lysates were obtained as previously described and protein concentration determined by the Bio-Rad assay. Lysates were immunoprecipitated with anti-PKCζ antibody, followed by Western analyses for phospho-Akt1 (p-Akt1).

C. Transfection of HEK 293 cells with PKCε constructs

HEK 293 cells were transfected with either wild-type or dominant-negative mutant PKCε (a generous gift from Dr. I. Bernard Weinstein) in an analogous manner as described for PKCζ. The wild-type construct is a full length PKCε in a pHACE vector, whereas the dominant-negative mutant construct is the full length PKCε with a point mutation in the catalytic domain at the ATP binding site. Transfection efficiency was consistently ~40%, as determined by green fluorescent protein co-transfection assay. Cells were pretreated with either C_6-

ceramide (1 μM) or DH-C_6-ceramide (1 μM) and then treated with IGF (50 ng/ml) for 5 minutes. Western blot analysis was performed, using lysates from either wild-type or dominant-negative PKCε construct transfected HEK cells, in order to determine the expression level of phospho-ERK (pERK). pERK bands were visualized with ECL. As a control, HEK cells were transfected with empty vector. To verify that the transfections with constructs for PKCε did not alter protein levels of other PKC isoforms, Western analyses were performed to assess PKCα, ε and ζ expression.

2.6 PROLIFERATION ASSAY

A. HEK 293: Cell Proliferation Assay

HEK 293 cells were grown to about 60% confluence in DMEM cell culture medium containing 10% fetal bovine serum in 12-wells cell culture plates. The HEK cells were then down-regulated by a 48 hour incubation in DMEM without fetal bovine serum. The cells were pretreated with either 1 μM C_6-ceramide, 1 μM dihydro-C_6-ceramide or vehicle for 1 hour and then treated with mitogens (IGF-1 or OAG) for an additional 18 hours. These treated HEK 293 cells were further incubated with 0.3 μCi/ml [^{3}H]-thymidine during the last 6 hours of treatment. The cells were washed once with ice-cold PBS and then washed 3 times for 5-10 minutes with 10% trichloroacetic acid. The fixed cells were then solubilized in 0.3M NaOH/ 0.1% SDS solution, and [^{3}H]-thymidine incorporation into acid-insoluble DNA was quantified by measuring radioactivity using a liquid scintillation counter.

B. HEK 293: Proliferating Cell Nuclear Antigen (PCNA) Expression

HEK 293 cells were grown to about 60% confluence in DMEM cell culture medium containing 10% fetal bovine serum in 12-wells cell culture plates. The HEK cells were then down-regulated by a 48 hour incubation in DMEM without fetal bovine serum. The cells were pretreated with 1 μM C_6-ceramide for 1 hour and then treated with mitogens, either IGF-1 or OAG, for 18 hours. Cells were then lysed and protein levels determined as previously described. HEK 293 lysates were then separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Western analyses was then preformed on the membranes in order to assess PCNA protein levels. Bands were detected by ECL®.

2.7 Statistical Analysis

The results were expressed as means ± SEM of 3 or more experiments. The data were analyzed by one-way ANOVA, followed by unpaired t-tests, corrected by the Bonferroni method. In selected experiments, non-parametric data were analyzed by either Mann-Whitney or Kruskal-Wallis one-way ANOVA tests, followed by Dunn's correction method. In those experiments where the control optical density (O.D.) values were set to 100%, the SEM for each of these control values was reported utilizing the non-transformed data.

Chapter 3

RESULTS

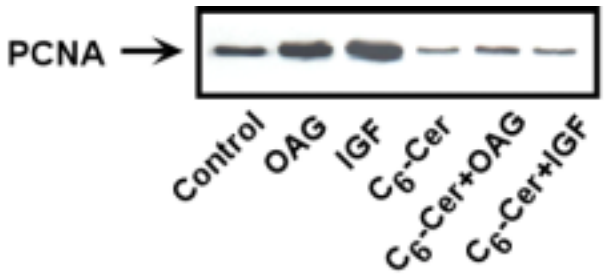
3.1 CELL PROLIFERATION EXPERIMENTS

Ceramide inhibits IGF-1 and OAG-stimulated HEK 293 cell growth.

The main hypothesis underlying these experiments is that ceramide inhibits cellular proliferation. Therefore, the ability of ceramide to inhibit cellular proliferation was initially assessed. One way to assess cells undergoing the cell cycle is to determine Proliferating Cell Nuclear Antigen (PCNA) protein levels. PCNA is a general marker of cells undergoing the cell cycle. As shown in **Figure 5**, both OAG (1 μM), the cell-permeable DAG mimetic, and IGF-1 (50 ng/ml) significantly increase PCNA expression by about 96% and 109%, respectively, compared to control cells. However, when the cells are pre-treated with C_6-ceramide (1 μM), the mitogens are no longer able to increase significantly PCNA expression. Thus, we concluded from these experiments that ceramide inhibits PCNA expression and, therefore, inhibits cells from undergoing the cell cycle.

Western analysis of PCNA expression is a crude method of assessing cellular proliferation. Therefore, to demonstrate conclusively the ability of ceramide to inhibit cellular proliferation, we performed [^{3}H]-thymidine incorporation studies, a standard technique for assessing proliferation. HEK 293 cells were treated with either IGF-1 (IGF-1) or 1-oleoyl-2-acetyl-glycerol (OAG), a cell-permeable mimetic of DAG, and [^{3}H]-thymidine uptake into acid-insoluble DNA was measured. As shown in **Figure 6**, both IGF-1 and OAG significantly

A



B

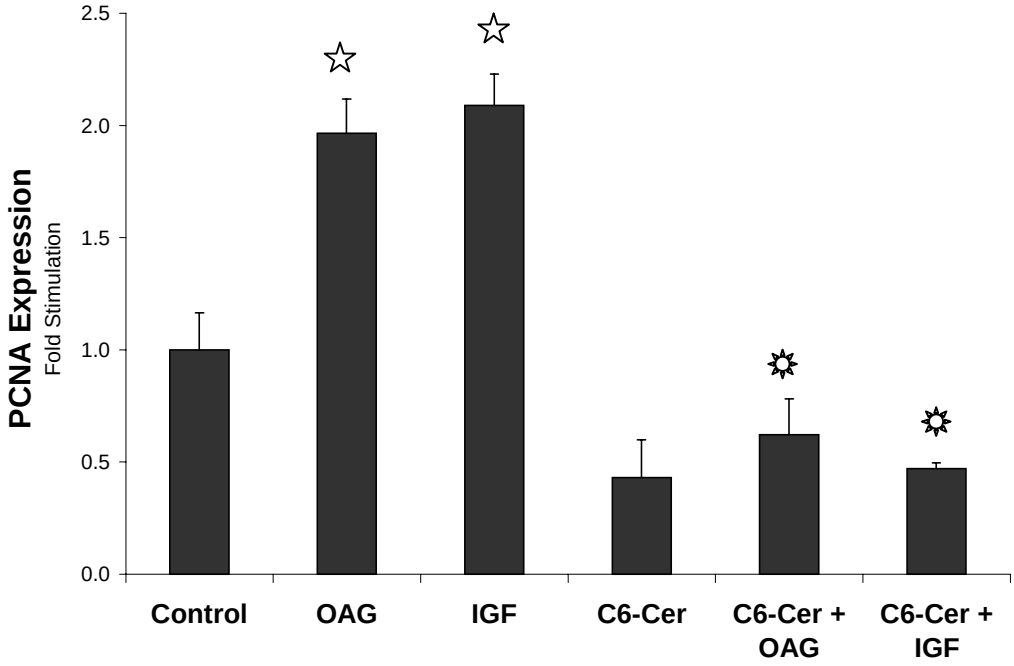


Figure 5. Ceramide decreases mitogen-stimulated PCNA expression in HEK 293 cells. Cell cycle progression was assessed by determining PCNA expression for quiescent cells treated for 18 hours with either IGF-1 or OAG. Pre-treatment for 1 hour with C_6-ceramide (C_6-Cer) reduces PCNA protein levels. This figure depicts the results obtained from n=4 experiments. **5A** depicts a representative Western blot. **5B** represents the averaged data from all experiments. Mean $\pm$ SEM. ☆ , significantly different from vehicle control; $p < 0.005$, unpaired Mann-Whitney test. ✡ , significantly different from IGF-1- and OAG-stimulated condition; $p < 0.0005$, unpaired Mann-Whitney test.

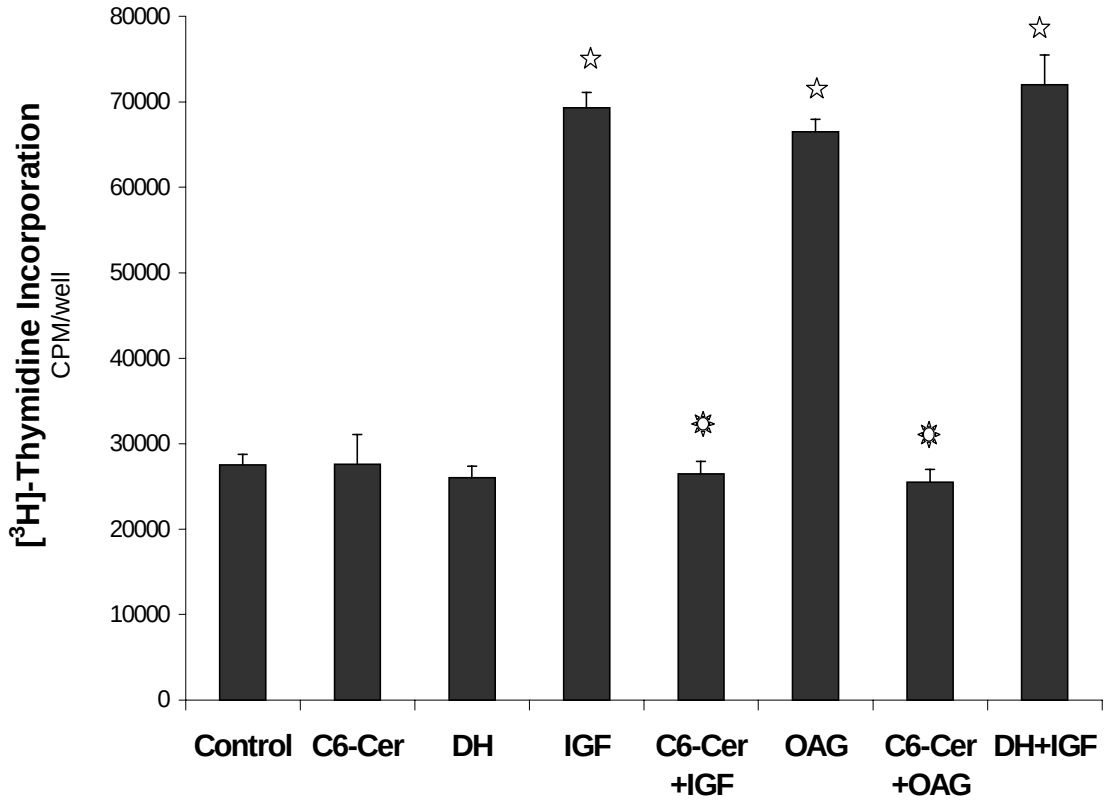


Figure 6. Ceramide reduces [^{3}H]-thymidine incorporation in HEK 293 cells. Cellular proliferation was assessed by means of incorporation of [^{3}H]-thymidine into acid-insoluble DNA in cells treated for 18 hours with either IGF-1 or OAG. Pre-treatment for 1 hour with C_6-ceramide (C_6-Cer), but not dihydro-C_6-ceramide (DH), reduces [^{3}H]-thymidine incorporation in mitogen-treated cells. This figure depicts the results obtained from n=4 experiments. Mean ± SEM. ☆, significantly different from vehicle control; $p < 0.005$, unpaired t-Test, ✡, significantly different from IGF-1- and OAG-stimulated condition; $p < 0.0001$, unpaired t-Test.

increased HEK 293 cell growth by approximately 150%, compared to control cells. When HEK 293 cells were pre-treated with C_6-ceramide (1 μM), we observed a significant decrease in cell growth in response to IGF-1 or OAG. Specifically, C_6-ceramide inhibited IGF-1-and OAG-induced cell growth to near basal levels. In contrast, the inactive ceramide analogue, dihydro-C_6-ceramide (1 μM), did not reduce IGF-stimulated [^{3}H]-thymidine incorporation.

These results were consistent with our previous studies, which demonstrated that ceramide inhibited rat glomerular mesangial and A7r5 vascular smooth muscle cell growth induced by mitogenic stimuli [Mandal *et al*., 1997]. This inhibitory effect of C_6-ceramide on HEK 293 cell growth does not appear to be caused by cell death since C_6-ceramide, at concentrations up to 100 μM, did not induce apoptotic or necrotic cell death, as assessed by LDH release (unpublished data). Collectively, these results demonstrate that bioactive ceramide potently inhibits HEK 293 cell growth induced by OAG and IGF-1, activators of PKC-dependent signaling pathways.

We have demonstrated the ability of ceramide to influence cellular phenotype. Therefore, we next investigated the biochemical pathways by which ceramide induces its physiological effects.

3.2 PKCε and ERK

Ceramide inhibits DAG-dependent PKCε bioactivity.

The fact that ceramide potently inhibited IGF-1- and OAG-stimulated HEK 293 cell growth strongly suggests a possible inhibitory role of ceramide in PKCε

activation, since IGF-1-induced mitogenesis is predominantly transduced through PKCε in a DAG-dependent manner [Bieberich *et al*., 2000]. Therefore, by performing *in vitro* reconstitution activity assays, we examined the ability of ceramide to affect directly and acutely the bioactivity of immunoprecipitated PKCε. The immunocomplexes were treated with physiological DAG (1,2-Diolein) and/or physiological ceramide ($C_{18:1}$). Bioactivity was assessed by resolving radiolabeled phosphorylation of histone IIIS. As shown in **Figure 7** (**A and B**), the bioactivity of PKCε in DAG-treated immunoprecipitates was significantly increased (3 fold) compared to the control immunocomplexes without DAG treatment. This result was consistent with previous observations demonstrating that DAG is required for PKCε activation [Uberall *et al*., 1999]. When DAG-treated immunocomplexes were challenged with addition of $C_{18:1}$-ceramide, bioactivity of PKCε was significantly decreased.

To verify further the inhibitory actions of physiological $C_{18:1}$-ceramide upon immunoprecipitated PKCε, we performed direct *in vitro* kinase assays using purified, recombinant PKCε (**Figure 7C**). In support of the immunoprecipitated PKCε kinase assay, physiological ceramide significantly reduced DAG-stimulated recombinant PKCε activity. In addition, the cell permeable C_6-ceramide mimicked the effect of physiological ceramide to diminish DAG-stimulated PKCε activity. Again, the inactive cell permeable ceramide analogue, dihydro-C_6-ceramide, had no significant effect on either basal or DAG-stimulated PKCε activity. These studies, utilizing recombinant PKC protein, confirm that bioactive ceramides directly inhibit DAG-stimulated PKCε activity.

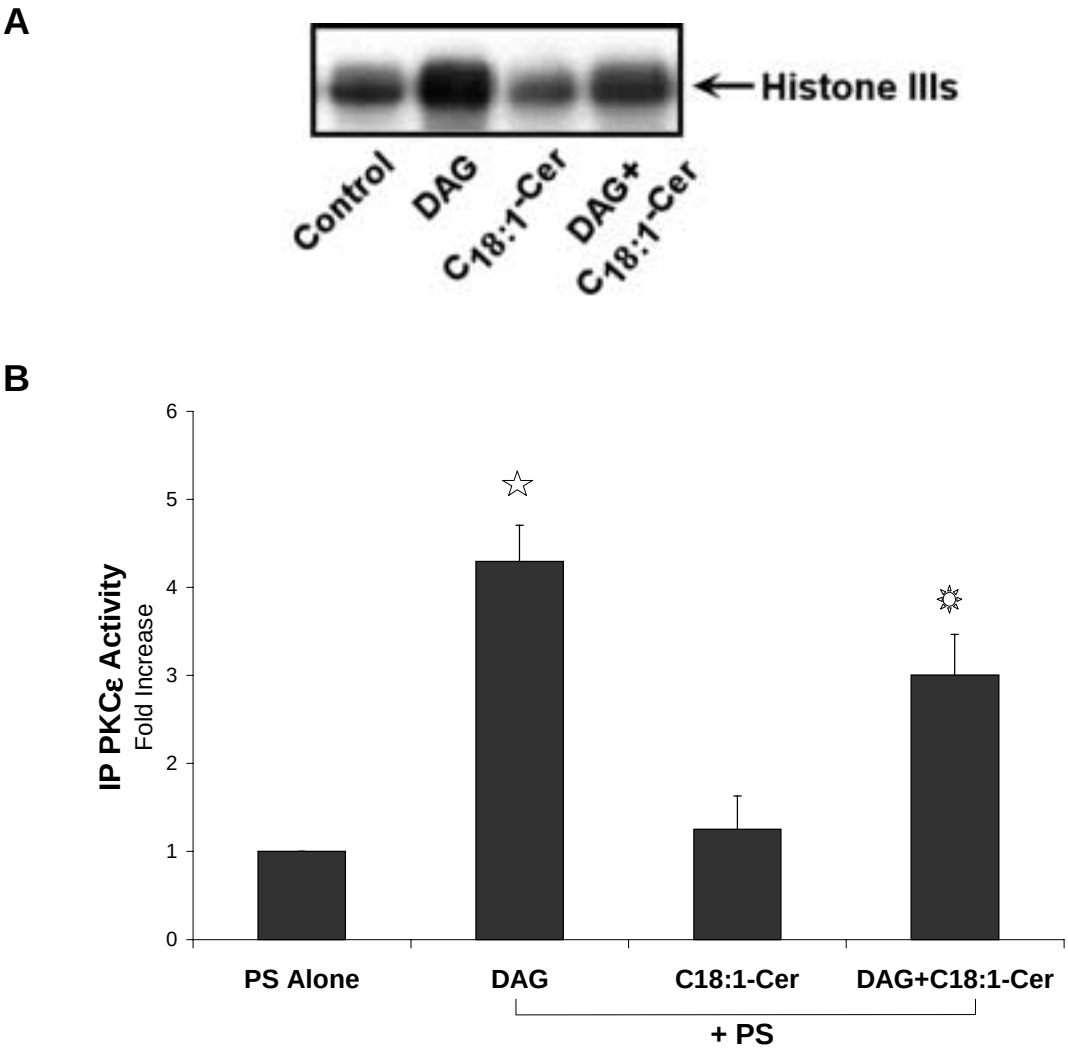


Figure 7. $C_{18:1}$-Ceramide directly inhibits DAG-stimulated immunoprecipitated and recombinant PKCε activity. PKCε immunocomplexes from HEK 293 cells were used in an *in vitro* reconstitution activity assay. Immunocomplexes were treated with either 1 μM 1,2 diolein (DAG) and/or 0.1μM $C_{18:1}$ ceramide for 20 minutes at 37°C. Phosphorylated histone IIIS protein was resolved on a 12% SDS-PAGE gel electrophoresis, visualized by autoradiography, and quantified by laser densitometry. Control experiments showed that histone phosphorylation was dependent on exogenous phosphatidylserine. **7A** depicts a representative autoradiogram of three such experiments. **7B** depicts the quantification of PKCε bioactivity. Mean ± SEM; n =3; ☆, significantly different from vehicle control; $p< 0.05$, unpaired Mann-Whitney test. ✡ , Combination treatment is significantly different from DAG treatment; $p < 0.05$, unpaired Mann-Whitney test.

C

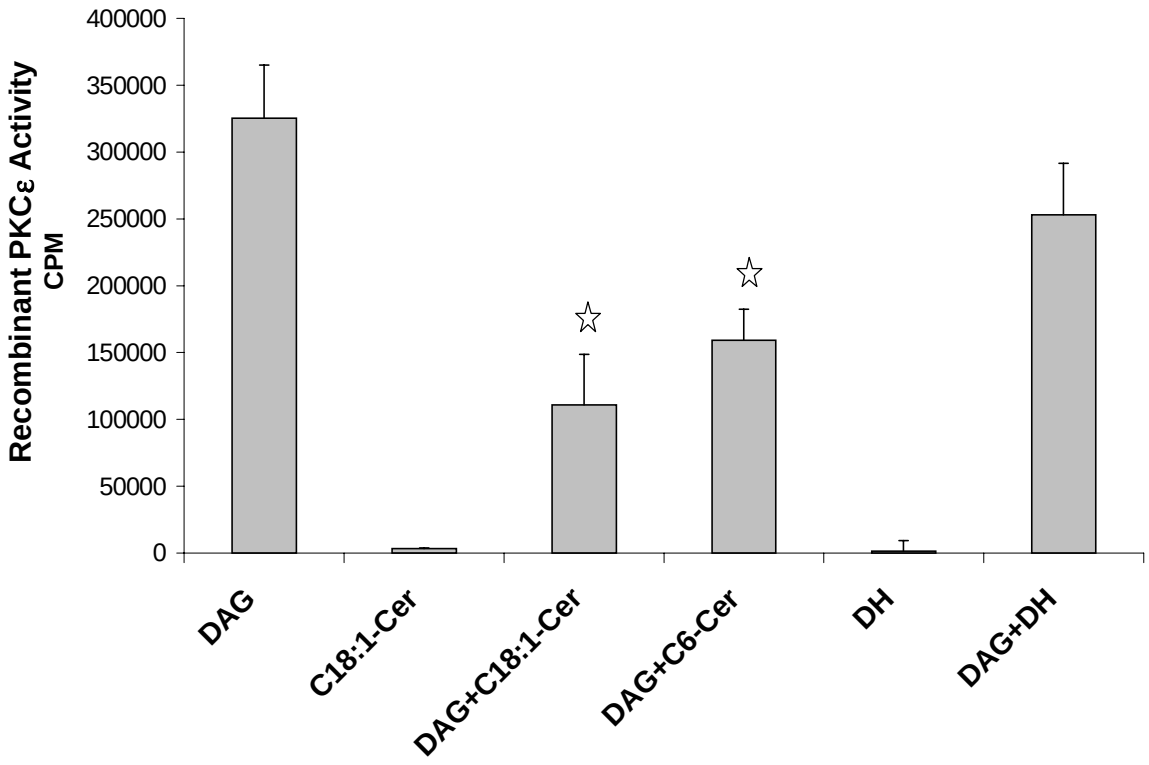


Figure 7. $C_{18:1}$-Ceramide directly inhibits DAG-stimulated immunoprecipitated and recombinant PKCε activity. Purified, recombinant PKCε (50 ng/ml) was used in an *in vitro* reconstitution activity assay. Samples were treated with either 1 μM 1,2 diolein (DAG) and/or 1 μM $C_{18:1}$ ceramide ($C_{18:1}$-Cer), 1 μM C_6-ceramide (C_6-Cer), 1 μM dihydro-C_6-ceramide (DH) for 15-20 minutes. Phosphorylated histone IIIS protein was resolved by a 12% SDS-PAGE gel electrophoresis and transferred to nitrocellulose. The phosphorylated histone bands were excised and quantified by liquid scintillation counting. Phosphatidylserine-induced PKCε activity was considered to be the basal level of activity. **7C** depicts the results of three similar experiments. Mean ± SEM; n = 3. ; ☆ significantly different from DAG treated; $p < 0.01$, unpaired t-Test.

To confirm these observations, additional experiments quantified phosphorylation of an alternative PKC substrate, myelin basic protein, by liquid scintillation analysis (**Table 1**). Again, using immunoprecipitated PKCε, physiological ceramide significantly inhibited DAG-stimulated phosphorylation of exogenous substrate. In contrast, the inactive ceramide analogue, dihydro-C_6-ceramide, did not inhibit DAG-stimulated PKCε activity. Collectively, these results further suggest an apparent reciprocal relationship between bioactive ceramide and DAG for PKCε bioactivity.

Ceramide does not change PKCε expression.

To determine whether inhibitory actions of ceramide on PKCε activity are also a consequence of altered protein expression, we examined PKCε protein expression by performing Western blot analysis using anti-PKCε antibody. As shown in **Figure 8**, when HEK 293 cells were treated with C_6-ceramide, protein expression of PKCε was not altered compared to control cells without ceramide treatment. These results demonstrate that ceramide treatment of HEK 293 cells does not alter PKCε protein expression. Furthermore, these results suggest that the inhibitory actions of ceramide may involve a direct inactivation of PKCε, and not down-regulation of protein expression.

PKCε is a necessary component for ceramide inhibition of ERK activity.

In order to confirm further that ceramide may exert its cell growth inhibitory actions through inactivation of PKCε, we examined the effects of C_6-ceramide in

Table 1. Immunoprecipitated PKCε activity using myelin basic protein as the exogenous substrate.

PKCε Activity		
Condition	- DAG	+ DAG
Control	4158 ± 805	10864 ± 1023*
$C_{18:1}$-Cer	5095 ± 578	7006 ± 35* #
Dihydro-Cer	4762 ± 244	11105 ± 646*

Means (**CPM**) + SEM; *n* = 3.
* Significantly different ($p<0.01$) from control condition
# Significantly different ($p<0.02$) from DAG-stimulated condition

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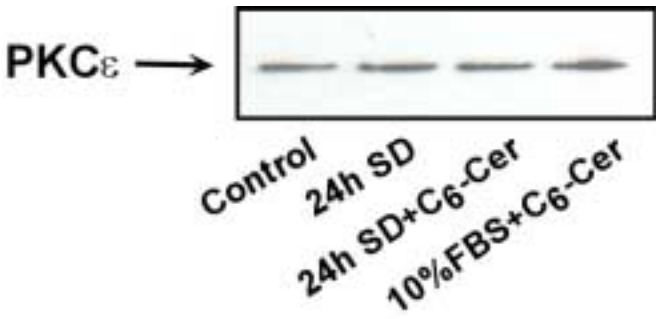

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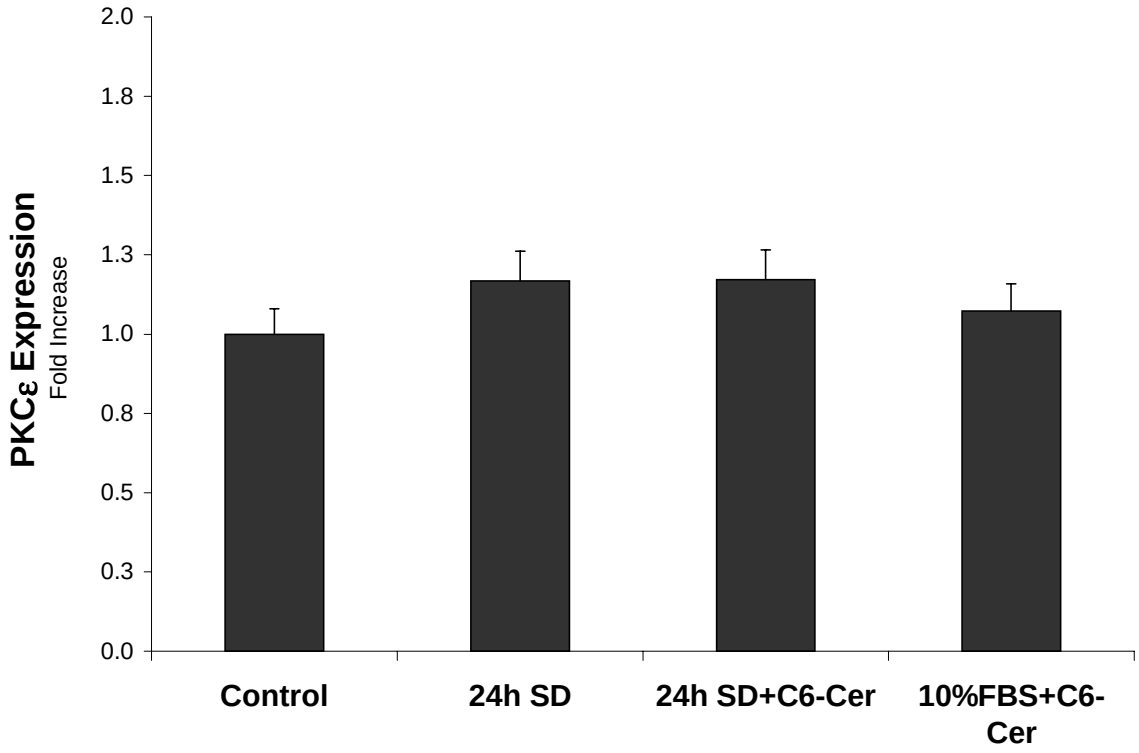

Figure 8. Neither Ceramide nor Serum Deprivation alters PKCε expression. Western analyses using polyclonal anti-PKCε antibodies are illustrated. HEK 293 cells cultured in 12-well culture plates in the presence or absence of 10% FBS were treated with either 1 μM C_6-ceramide or 0.01% DMSO vehicle for 24 hours. The protein bands were revealed using enhanced chemiluminescence. **8A** depicts a representative Western blot. **8B** graphically illustrates the quantification of the PKC bands. Mean ± SEM; n = 3; $p > 0.05$, Kruskal-Wallis test. The abbreviations used are: SD (Serum Deprivation); C_6-cer (Ceramide).

HEK 293 cells over-expressing dominant negative PKCε (ΔPKCε). Since the activation of ERK is required for IGF-1-induced mitogenesis, we initially investigated the involvement of PKCε in the ERK cascade through the use of wild type (WT) and ΔPKCε mutants. In data not shown, transfection with WT and ΔPKCε constructs resulted in equal expression of PKCε. However, both of these constructs had a higher level of PKCε expression compared to empty vector controls. In contrast to PKCε, cellular levels of other PKC isoforms, including α and ζ, did not change after transfection with any cDNA construct.

As shown in **Figure 9**, HEK 293 cells over-expressing WT PKCε showed significantly increased ERK bioactivity, as assessed by phosphorylated ERK (pERK), in response to IGF-1 treatment. Yet, HEK 293 cells over-expressing ΔPKCε showed significantly reduced IGF-induced ERK bioactivity by approximately 50%, which correlated with the determined transfection efficiency. When cells were treated with cell permeable ceramide in the presence of IGF, the bioactivity of ERK was substantially reduced in HEK 293 cells over-expressing WT PKCε, whereas the bioactivity of ERK in ΔPKCε over-expressing HEK 293 cells was not significantly changed. Furthermore, dihydro-C_6-ceramide-treated wild type-PKCε- or ΔPKCε expressing cells did not manifest decreased pERK expression in the presence of IGF-1. These results demonstrate that PKCε is a necessary signaling component for modulating ceramide-mediated inhibition of ERK bioactivity. In addition, these results suggest a role of ceramide in modulation of PKCε interaction with elements of the ERK signaling cascade.

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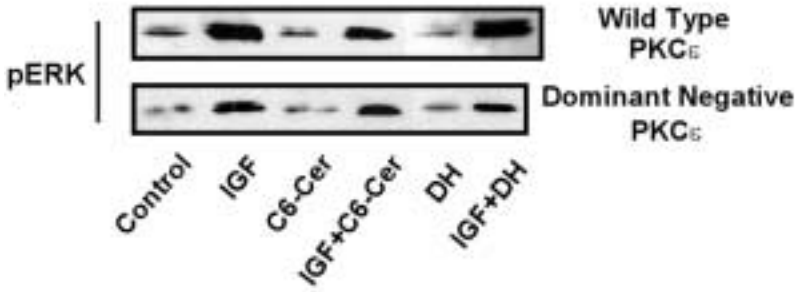

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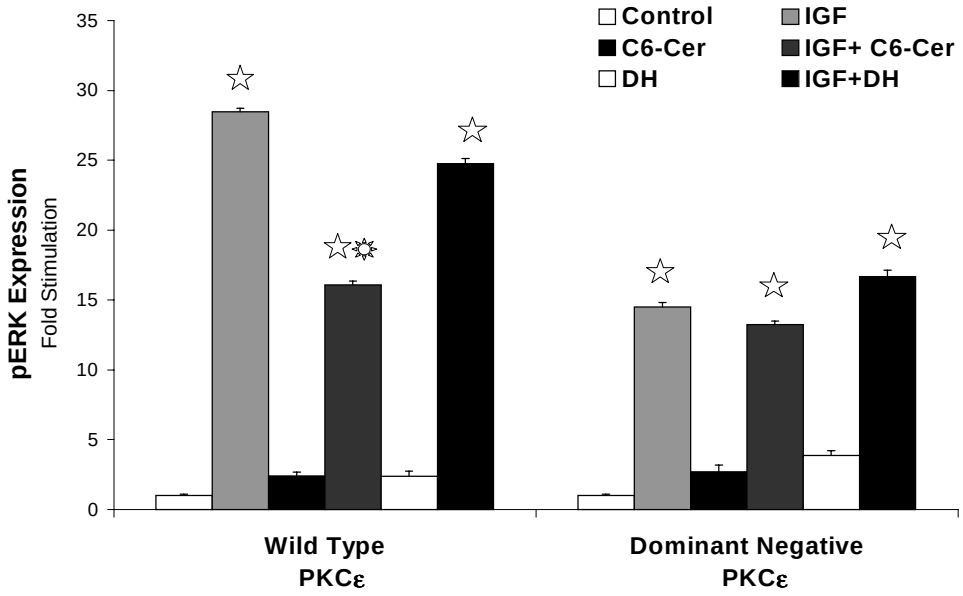

Figure 9. IGF-stimulated ERK bioactivity was reduced in HEK 293 cells over-expressing a PKCε dominant negative mutant. Western analyses utilizing an anti-phospho-ERK antibody were performed on HEK 293 cells over-expressing either a wild type or dominant negative mutant form of PKCε. Cells were treated with IGF-1 (50 ng/ml), C_6-ceramide or dihydro-C_6-ceramide (1 μM) or a combination treatment for 5-10 minutes. The expression of a dominant negative form of PKCε mimicked the effect of ceramide to reduce IGF-1-induced ERK bioactivity. Data was normalized to the appropriate control. Cells transfected with empty vector had a similar pattern of pERK expression as cells over-expressing wild type PKCε (data not shown). **9A** depicts representative Western blots for the PKCε mutants. **9B** graphically illustrates the quantification of the pERK bands. Mean ± SEM; n = 5; ☆, significantly different from vehicle control; $p < 0.001$, unpaired Mann-Whitney test. ✡ , Combination treatment is significantly different from individual treatment alone; $p < 0.02$, unpaired Mann-Whitney test.

Ceramide inhibits PKCε•ERK interaction.

As PKCε activation has been shown to be an upstream kinase of ERK activation [Hurley *et al*., 1997; Kim *et al*., 1991], we performed co-immunoprecipitation assays to determine whether ceramide can inhibit the ability of PKCε to interact with ERK, resulting in a decreased ERK activity. Since ceramide has also been shown to activate PKCζ [van Blitterswijk, 1998], we also investigated whether ceramide regulates PKCζ•ERK interaction. As shown in **Figure 10**, HEK 293 cells treated with IGF-1 specifically increased PKCε, but not PKCζ, association with ERK2. C_6-ceramide treatment abrogated this IGF-1-induced interaction between ERK and PKCε. By itself, C_6-ceramide exerted no significant effect on these interactions. These results demonstrate that ceramide specifically precludes the IGF-1-induced interaction between PKCε and ERK2.

Ceramide inhibits PKCε association with pERK.

Possibly only activated PKCε may recruit and activate ERK through phosphorylation. Therefore, the inactivation of PKCε may result in blocked ERK recruitment and subsequent activation. To determine whether ceramide specifically blocks PKCε interaction with phosphorylated/activated ERK, we performed co-immunoprecipitation assays between PKCε and pERK. As shown in **Figure 11**, IGF-1 treatment significantly increased (6-fold) the association of PKCε with pERK in HEK 293 cells. C_6-ceramide, but not dihydro-C_6-ceramide, pretreatment significantly reduced IGF-1-stimulated PKCε association with pERK.

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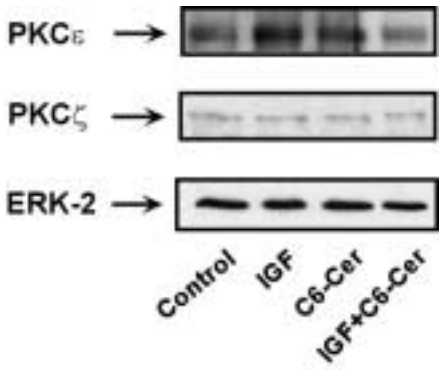

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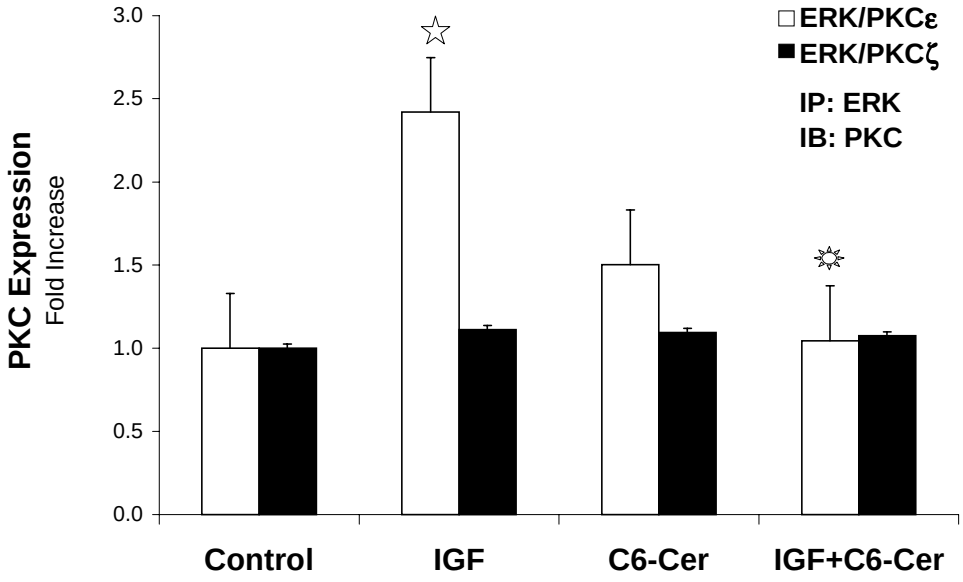

Figure 10. Ceramide reduces PKCε interaction with ERK. ERK2 immunocomplexes of HEK 293 cells were used to assess protein interaction between select PKC isoforms and ERK. The cells were treated with DMSO vehicle control, IGF-1 (50 ng/ml), C_6-ceramide (1 μM) or IGF-1 plus C_6-ceramide for 5-10 minutes. PKC isoforms that co-immunoprecipitated with anti-ERK2 antibody were detected by ECL. C_6-Ceramide diminished IGF-1 induced interactions of PKCε, but not PKCζ with the ERK2 pathway. **10A** depicts representative Western blots for the PKC and ERK-2. **10B** graphically illustrates the quantification of the PKC bands. Mean ± SEM; n=4;☆, significantly different from control; $p<0.05$, unpaired Mann-Whitney test. ✡, Combination treatment is significantly different than either treatment alone; $p<0.01$, unpaired Mann-Whitney test.

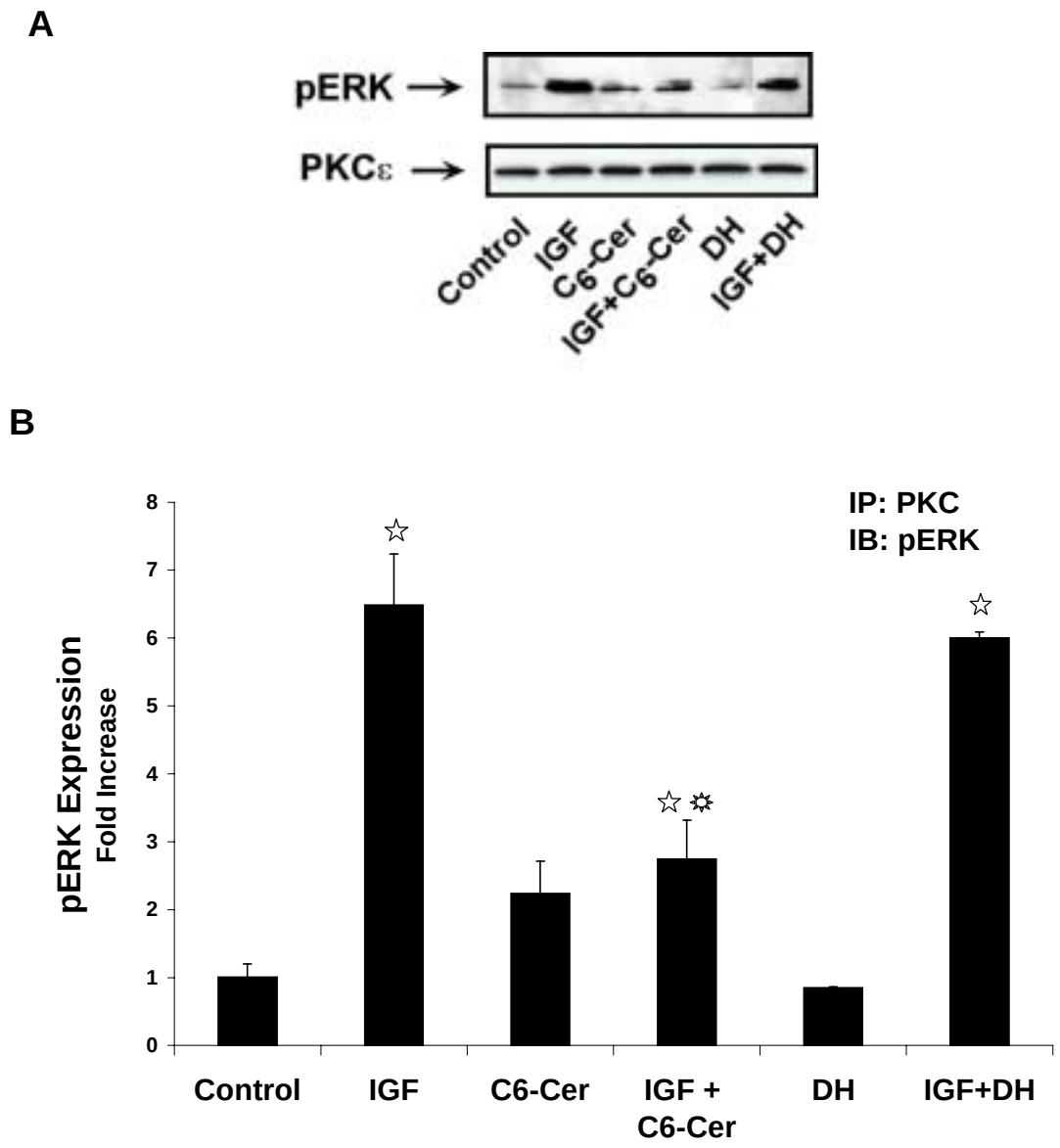

Figure 11. Ceramide inhibits ERK activity in PKCε immunoprecipitates. HEK 293 cells were used to assess PKCε interaction with bioactive phospho-ERK. HEK 293 cells were treated with IGF-1 (50 ng/ml) and/or C_6-Ceramide (C_6-Cer, 1 μM) for 5-10 minutes. The lysates from PKCε immunocomplexes were probed with an antibody that detects pERK. C_6-ceramide pretreatment led to a significant reduction in the IGF-1-stimulated PKCε-associated increase in ERK bioactivity. Positive and negative controls included whole cell lysates and cell-free samples, respectively (data not shown). **11A** depicts a representative autoradiogram of three such experiments. **11B** depicts the quantification of PKCε bioactivity. Mean ± SEM; n=3-4; ☆, significantly different from vehicle control; $p<0.01$, unpaired Mann-Whitney test. ✡ , Combination treatment is significantly different from individual treatment alone; $p < 0.001$, unpaired Mann-Whitney test.

Changes in pERK expression most likely reflect agonist-regulated interactions between PKCε and pERK as equal levels of PKCε were observed in the immunoprecipitates from all treatments. These results demonstrate that bioactive ceramide specifically inhibits PKCε interaction with pERK.

Ceramide inhibits PKCε interaction with Raf-1.

We demonstrated that ceramide blocked ERK activation via selective inhibition of PKCε activity. However, it is not clear whether selective ceramide inhibition of ERK activity is dependent on Raf-1, an upstream kinase of ERK. Others have demonstrated both Raf-1-dependent and-independent activation of ERK by PKCs. Therefore, we examined whether Raf-1 kinase is co-immunoprecipitated with PKCε in HEK 293 cells treated with IGF-1. As shown in **Figure 12**, we observed a strong association of PKCε with Raf-1 in response to IGF-1 treatment. This result was consistent with other reports [Mathias *et al*., 1993], which demonstrated that PKCε associates with Raf-1 and that this association is increased by growth factor treatment. In contrast, HEK 293 cells treated with either C_6-ceramide or IL-1, a receptor-mediated inducer of ceramide formation [Coroneos *et al*., 1996], do not induce association of PKCε with Raf-1. In fact, IGF-1-stimulated Raf-1 association with PKCε is significantly inhibited by pretreatment with C_6-ceramide or IL-1, demonstrating that ceramide potently inhibits IGF-1-stimulated PKCε interaction with Raf-1. Collectively, these data support our conclusion that direct inhibition of PKCε by ceramide inhibits the

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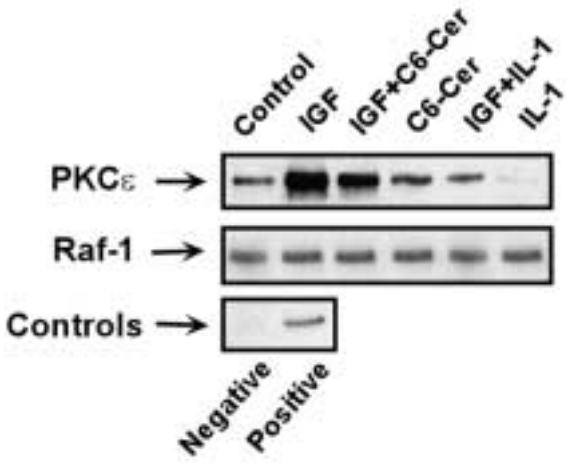

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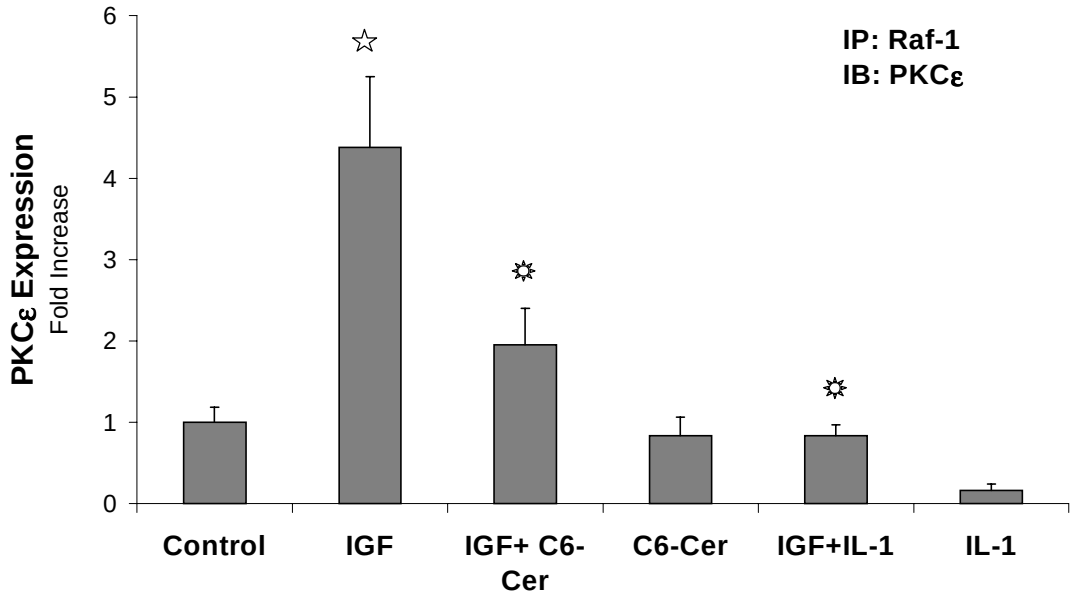

Figure 12. Ceramide decreases the IGF-1-stimulated association between PKCε and Raf-1. HEK 293 cells were used to assess PKCε interaction with Raf-1. Cells were treated with IGF-1 (50 ng/ml) and/or C_6-Ceramide (C_6-Cer, 1μM) and/or IL-1 (20 ng/ml) for 5-10 minutes. Pretreatment with ceramide or IL-1 resulted in a decreased IGF-1-induced association of Raf-1 with PKCε. Negative and positive controls included cell-free samples and whole cell lysates, respectively. **12A** depicts a representative experiment. **12B** depicts the quantification of PKCε bands. Mean ± SEM; n = 4; ☆, significantly different from vehicle control; $p < 0.01$, unpaired Mann-Whitney test. ✡, Combination treatment is significantly different from individual treatment alone; $p < 0.01$, unpaired Mann-Whitney test.

interaction between PKCε and upstream elements of the ERK cascade (**Figure 13**).

3.3 PKCζ and SAPK

Ceramide directly stimulates PKCζ bioactivity.

Ceramide has been shown to activate the SAPK cascade. However, the mechanism of activation has not been clearly defined. As PKCζ has one cysteine rich domain (CRD), which is suggested to interact with ceramide, but not diacylglycerol (DAG) [van Blitterswijk, 1998], we hypothesize that ceramide-activated PKCζ regulates the SAPK cascade. Therefore, the ability of ceramide to affect the directly and acutely bioactivity of immunoprecipitated PKCζ from HEK 293 cells was assessed by performing *in vitro* reconstitution activity assays. The immunocomplexes were treated for 15 minutes with physiological ceramide ($C_{18:1}$-ceramide) and/or physiological DAG (1,2-Diolein). Bioactivity was assessed by resolving radiolabeled phosphorylation of Histone IIIS. As shown in **Figure 14A and 14B**, the bioactivity of immunoprecipitated PKCζ was significantly increased (3 fold) by ceramide, but not by DAG treatment. When $C_{18:1}$-ceramide-treated immunocomplexes were challenged with the addition of DAG, the bioactivity of PKCζ was significantly decreased. These results suggest an apparent reciprocal relationship between ceramide and DAG for PKCζ bioactivity.

To verify the stimulatory actions of physiological $C_{18:1}$-ceramide upon immunoprecipitated PKCζ, we performed direct *in vitro* kinase activity assays

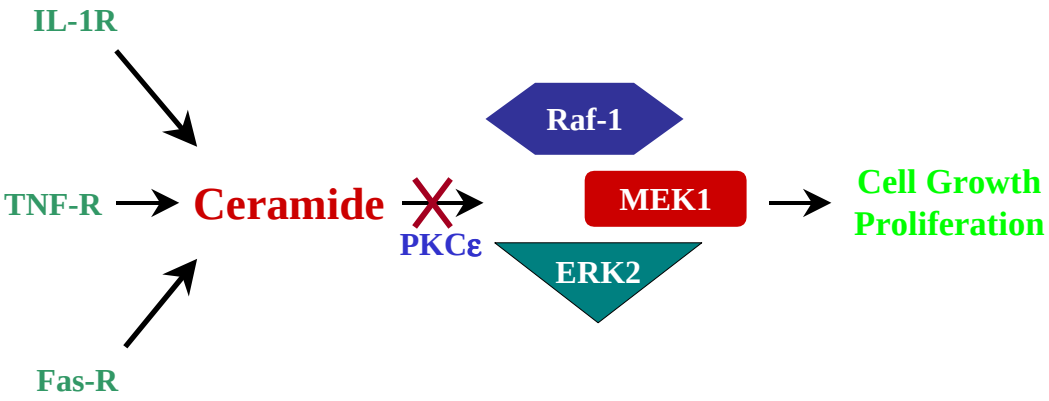

Figure 13. Mechanism by which ceramide inhibits the ERK cascade.

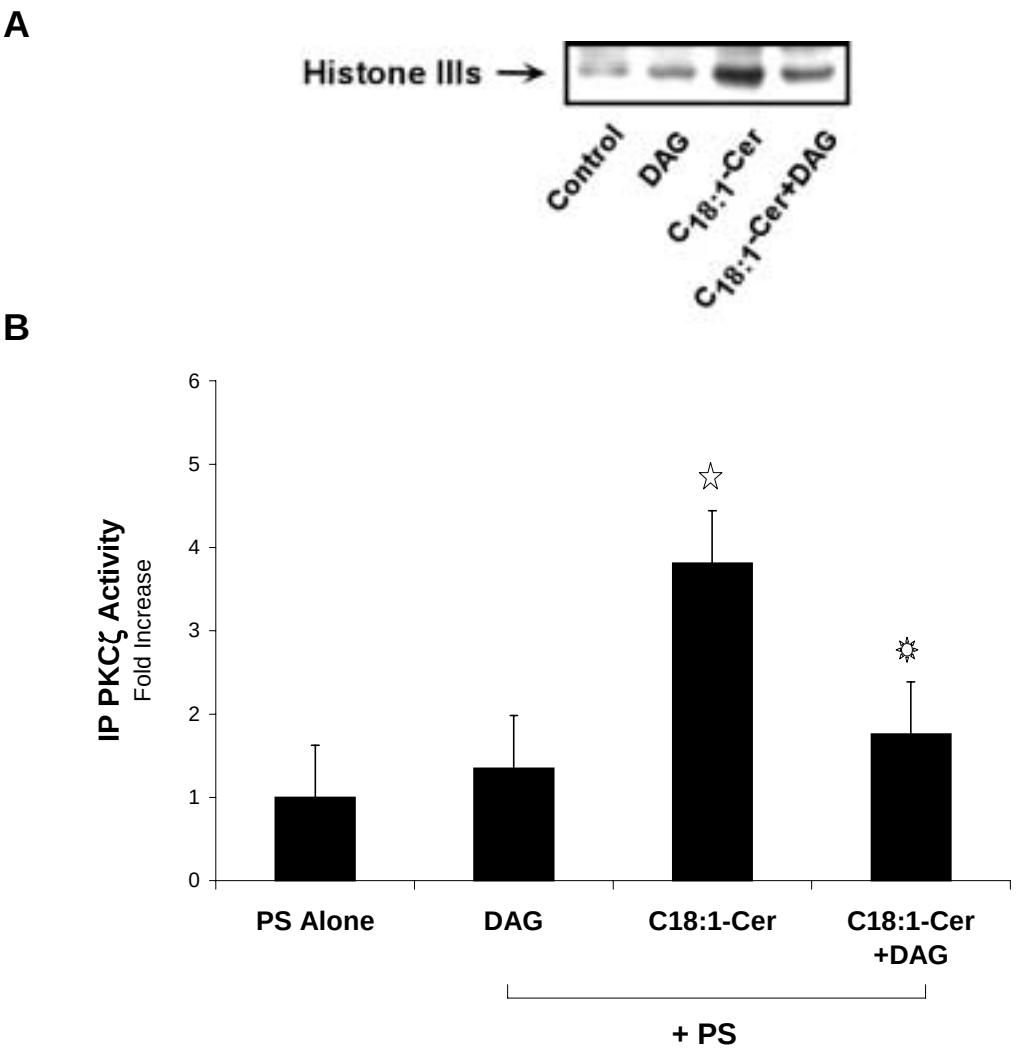

Figure 14. $C_{18:1}$-Ceramide directly stimulates immunoprecipitated and recombinant human PKCζ activity. For the immunoprecipitated PKCζ kinase assays, phosphorylated histone IIIS protein was resolved on a 12% SDS-PAGE gel electrophoresis and visualized by autoradiography. For immunoprecipitated PKCζ, $C_{18:1}$-ceramide directly stimulated PKCζ activity. DAG itself had no effect upon either immunoprecipitated PKCζ but significantly reduced $C_{18:1}$-ceramide-induced PKCζ activity. Results similar to $C_{18:1}$-ceramide were observed for the cell-permeable C_6-Ceramide (data not shown). Control experiments showed that histone phosphorylation was dependent on exogenous phosphatidylserine (PS) (data not shown). **14A** depicts a representative autoradiogram for the immunoprecipitated PKCζ assay. **14B** depicts the quantification of the immunoprecipitated PKCζ bioactivity. Mean ± Standard Error; n=3-4; ☆, significantly different from vehicle control; $p < 0.01$, unpaired Mann-Whitney test , ✡, Combination treatment is significantly different from individual treatment alone; $p < 0.01$, unpaired Mann-Whitney test.

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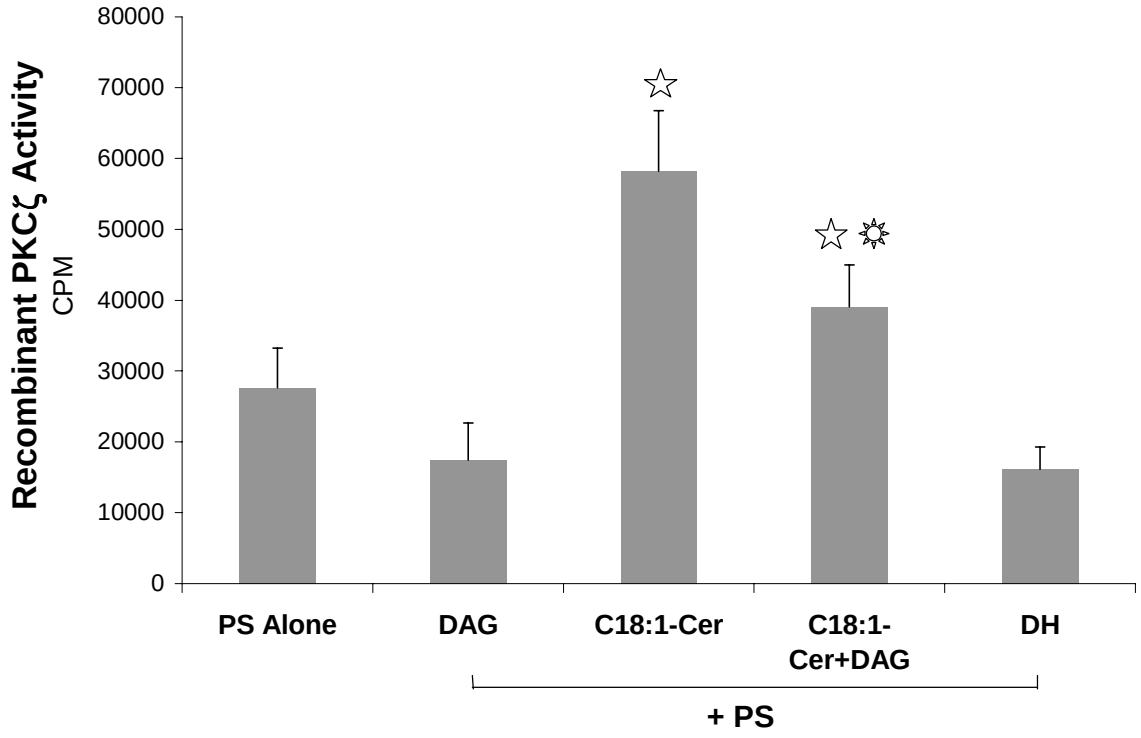

Figure 14. $C_{18:1}$-Ceramide directly stimulates immunoprecipitated and recombinant human PKCζ activity. Purified recombinant PKCζ (50 ng) (**14C**) was used in an *in vitro* reconstitution activity assay. The recombinant PKCζ was treated with either 10^{-6} M 1,2 diolein (DAG) and/or 10^{-6} M $C_{18:1}$ ceramide ($C_{18:1}$-Cer), as well as dihydro-C_6-ceramide (DH), for 15 minutes at 37°C. Control experiments showed that histone phosphorylation was dependent on exogenous phosphatidylserine (PS) (data not shown). For the recombinant protein assays, the phosphorylated histone bands were excised and quantified by liquid scintillation counting. Recombinant PKCζ, $C_{18:1}$-ceramide directly stimulated PKCζ activity. DAG itself had no effect upon recombinant PKCζ but significantly reduced $C_{18:1}$-ceramide-induced PKCζ activity. Results similar to $C_{18:1}$-ceramide were observed for the cell-permeable C_6-Ceramide (data not shown). For the recombinant PKCζ assay, the inactive ceramide analogue, DH-C_6-ceramide, did not increase PKCζ activity. **14C** depicts the quantification of the recombinant PKCζ assays. Mean ± Standard Error; n=3-4; ☆ , significantly different from vehicle control; $p < 0.01$, unpaired t-Test. ✡ , Combination treatment is significantly different from individual treatment alone; $p < 0.01$, unpaired t-Test.

using purified, recombinant PKCζ (**Figure 14C**). Consistent with the immunoprecipitated PKCζ kinase assay, physiological ceramide significantly activated recombinant PKCζ activity. Similar to results obtained in the immunoprecipitated PKC kinase assay, DAG-treatment had no effect by itself but was able to reduce $C_{18:1}$-ceramide-induced PKCζ activity. In addition, cell permeable C_6-ceramide mimicked physiological $C_{18:1}$-ceramide by stimulating PKCζ activity in cell-free assays (data not shown). In support of the specificity of the actions of ceramide upon PKCζ, the inactive cell permeable ceramide analogue, dihydro-C_6-ceramide, had no effect on PKCζ activity. These studies, utilizing both immunoprecipitated and recombinant human PKCζ protein, confirm the hypothesis that bioactive ceramides directly activate PKCζ activity.

Ceramide does not change PKCζ expression.

In addition to activating directly PKCζ, ceramide could also increase PKCζ activity by inducing PKCζ protein expression. We examined the PKCζ protein expression by performing Western blot analyses using anti-PKCζ antibody. As shown in **Figure 15**, when cycling (10% FBS) or non-cycling (Serum Deprived, SD) HEK 293 cells were treated with cell-permeable C_6-ceramide for 24 hours, protein expression of PKCζ was not altered compared to control cells without C_6-ceramide treatment. These results demonstrate that ceramide regulates HEK 293 cells as a consequence of direct PKCζ activation and not by up-regulating PKC protein expression.

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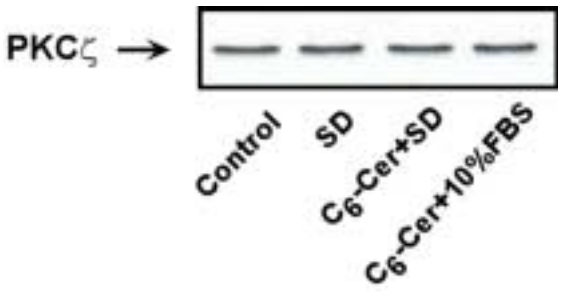

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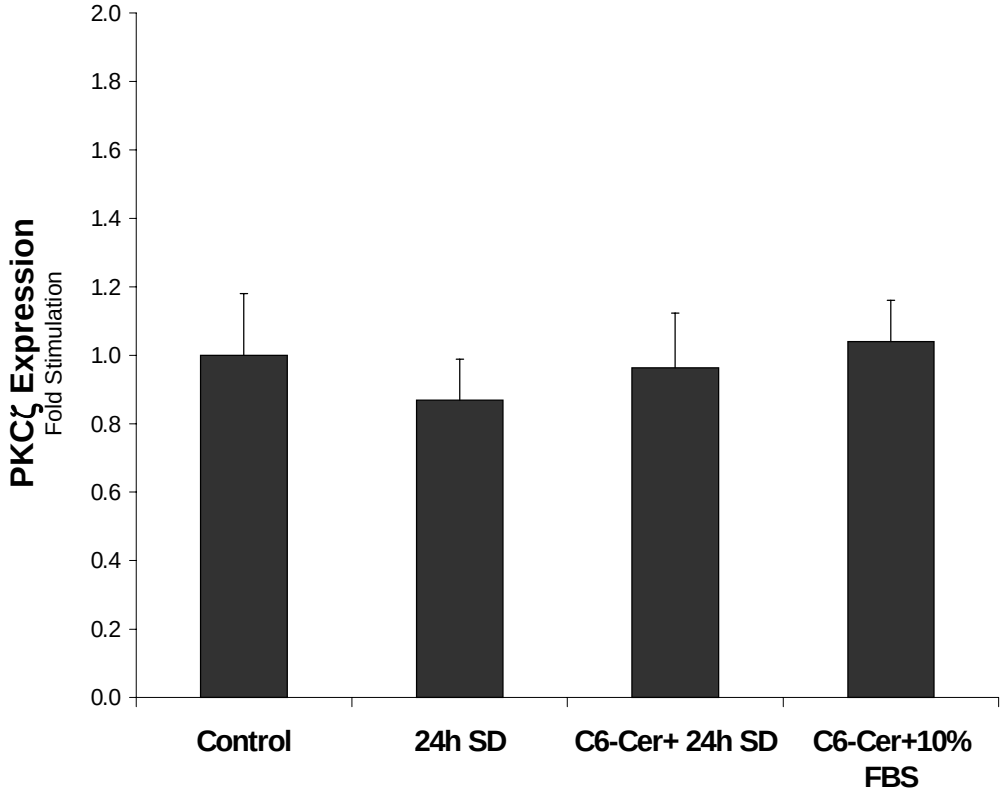

Figure 15. Neither Ceramide nor Serum Deprivation alters PKCζ expression. Western analyses using a polyclonal PKCζ antibody are illustrated. HEK-293 cells in the presence or absence of 10% FBS were treated with either 10^{-6}M C_6-Ceramide or 0.01% DMSO vehicle for 24 hours. The protein bands were revealed using enhanced chemiluminescence. No significant changes in PKCζ protein expression were observed among all conditions tested. **15A** depicts a representative Western blot. **15B** graphically illustrates the quantification of the PKCζ bands. Mean $\pm$ Standard Error; n=3; $p > 0.05$, Kruskal-Wallis test. The abbreviations used are: SD (Serum Deprivation); C_6-Cer (C_6-Ceramide).

PKCζ is a necessary component for ceramide activation of SAPK activity.

As we demonstrated that ceramide directly activates PKCζ, we examined whether ceramide-induced SAPK activity is dependent on PKCζ. As shown in **Figure 16**, HEK 293 cells over-expressing the WT PKCζ construct resulted in an increase in C_6-ceramide-stimulated immunoprecipitated SAPK bioactivity. In contrast, C_6-ceramide-induced SAPK activity was blocked with a dominant-negative mutant of PKCζ. These results suggest that PKCζ is a necessary signaling component for modulating ceramide-mediated activation of SAPK bioactivity.

Ceramide augments PKCζ•SAPK interaction.

To further define the mechanism by which ceramide activates PKCζ leading to SAPK complex formation, we next examined whether PKCζ associates with SAPK. Therefore, to document whether ceramide induces a potential interaction between PKCζ and SAPK, we performed co-immunoprecipitation assays. As shown in **Figure 17**, HEK 293 cells treated with C_6-ceramide specifically increased PKCζ association with SAPK1. Since ceramide induces the translocation of PKCε from the plasma membrane to the cytosol, an event consistent with inactivation [Sawai *et al*., 1997], we also investigated if ceramide regulates PKCε•SAPK interaction as a negative control. Ceramide did not augment an association between PKCε and SAPK. These results demonstrate that ceramide specifically induces an interaction between PKCζ and SAPK.

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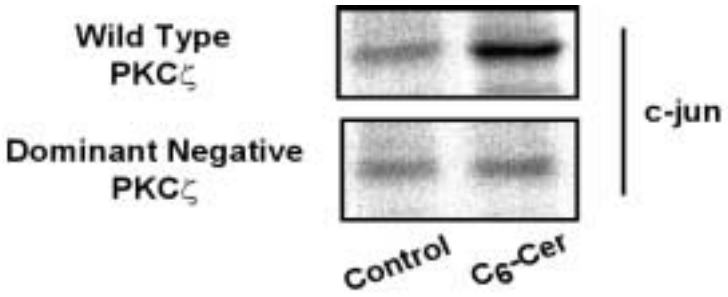

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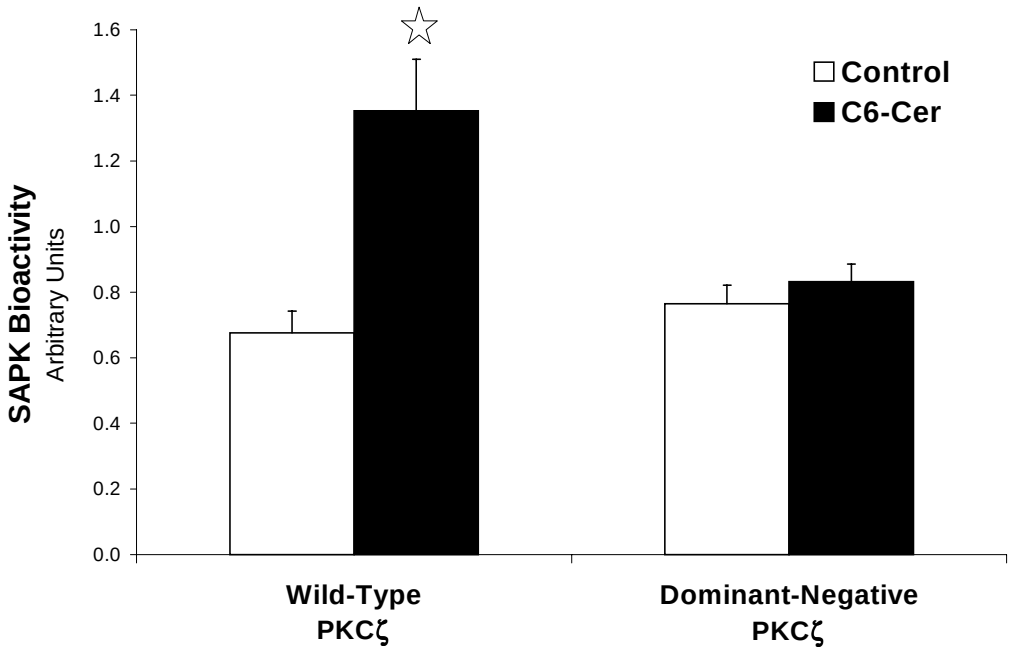

Figure 16. PKCζ is a necessary component for ceramide activation of SAPK activity. HEK 293 cells were transfected with wild type or dominant-negative PKCζ constructs. Transfected cells were treated with vehicle (0.01% DMSO) or 1 μM C_6-ceramide, lysed and immunoprecipitated with anti-SAPK1 antibody. An *in vitro* kinase assay was then performed on the immunocomplexes using c-jun as a phosphorylation substrate. As compared to over-expressed wild type PKCζ, expression of the dominant-negative form of PKCζ inhibited the ability of ceramide to stimulate SAPK bioactivity. **16A** depicts representative autoradiograms for the PKCζ mutants. **16B** graphically illustrates the quantification of the c-jun bands. Mean ± Standard Error; n=3;☆, significantly different from control; $p < 0.01$, unpaired t-Test.

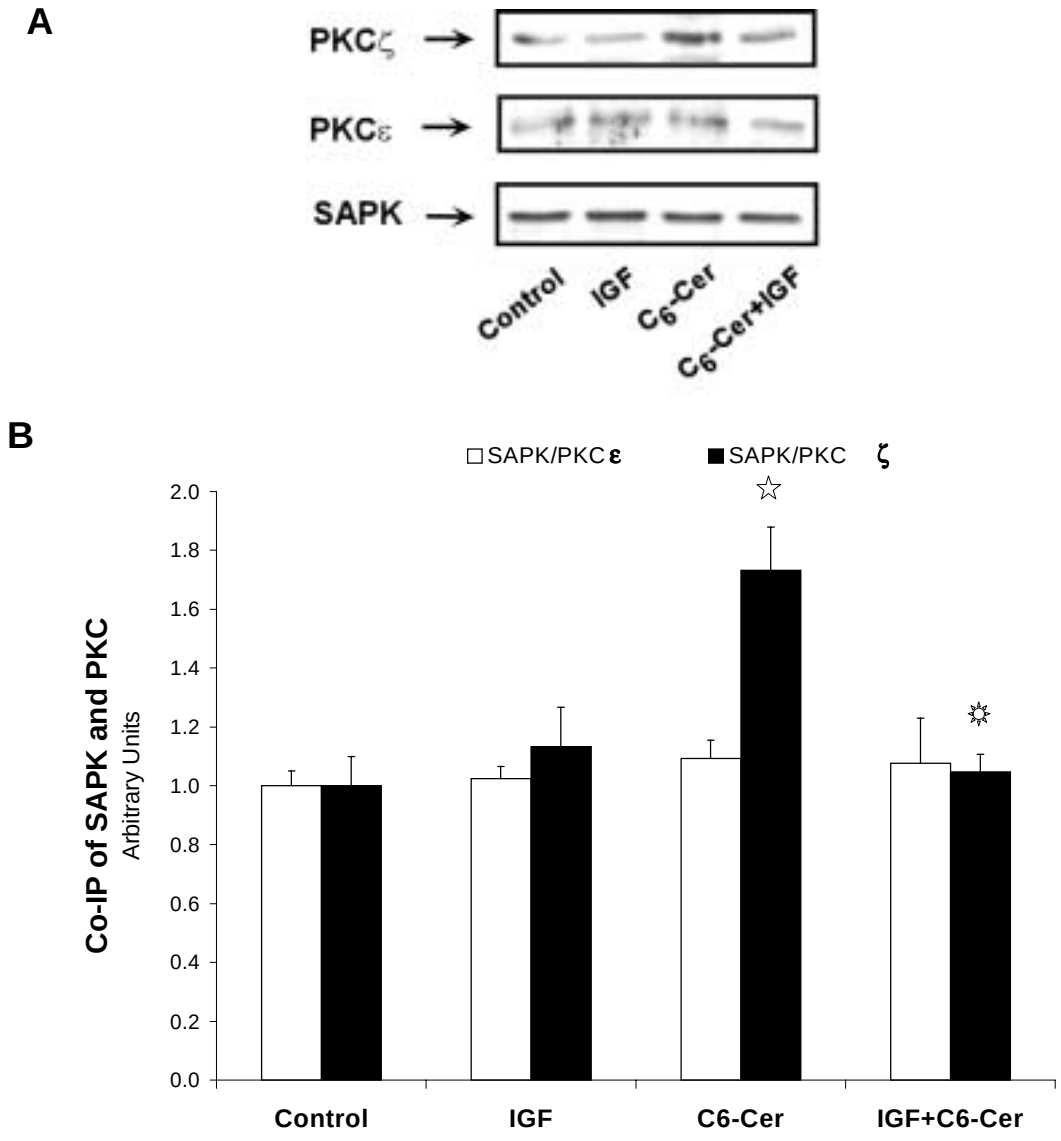

Figure 17. Ceramide enhances PKCζ interaction with SAPK1. Protein interactions between select PKC isoforms and SAPK were assessed by Western analyses utilizing anti-PKCζ or -PKCε antibodies on SAPK1 immunoprecipitates from HEK-293 cells. Cells were then treated with vehicle control (0.01% DMSO), IGF-1 (50 ng/ml), C_6-ceramide (10^{-6} M) or IGF plus C_6-ceramide for 5 minutes. C_6-Ceramide enhanced interactions of PKCζ, but not PKCε, with the SAPK1 pathway. **17A** depicts representative Western blots. **17B** graphically illustrates the quantification of the PKC bands. Mean ± Standard Error; n=3;☆, significantly different from vehicle control; $p < 0.01$, unpaired t-Test.✡, Combination treatment is significantly different from individual treatment alone; $p < 0.005$, unpaired t-Test.

IGF-1 induces DAG generation, which we have previously shown to inhibit ceramide-activated PKCζ. Thus we investigated whether IGF-1 could diminish ceramide-induced PKCζ•SAPK interactions. Our studies document that IGF-1, in contrast to ceramide, does not induce PKCζ association with SAPK. In fact, ceramide-induced association between SAPK and PKCζ was diminished. These data further support the specificity of ceramide-activated PKCζ to form SAPK signaling complexes.

Ceramide induces PKCζ association with p-SEK and MEKK1.

Our results imply a role for ceramide in modulating PKCζ interactions with upstream elements of the SAPK signaling cascade. Activated SEK is the immediate upstream dual specificity kinase that phosphorylates SAPK on threonine and tyrosine residues. It is possible that bioactive PKCζ may recruit and activate SEK through phosphorylation on Ser219 and Thr223. Therefore, by co-immunoprecipitation assays, we investigated the ability of ceramide to induce an association between PKCζ and bioactive (phosphorylated)-SEK (p-SEK) ceramide to induce an association between PKCζ and bioactive (phosphorylated)-SEK (p-SEK). As shown in **Figure 18A** and **18B**, C_6-ceramide or IL-1 treatment significantly increased (5-fold) the association of PKCζ with p-SEK in HEK 293 cells. Consistent with the effect of IGF-1 upon ceramide-induced PKCζ•SAPK interaction, IGF-1 treatment also reduced both C_6-ceramide and IL-1 induced association of PKCζ with p-SEK. These results demonstrate that ceramide regulates the activity of PKCζ and its interaction with p-SEK.

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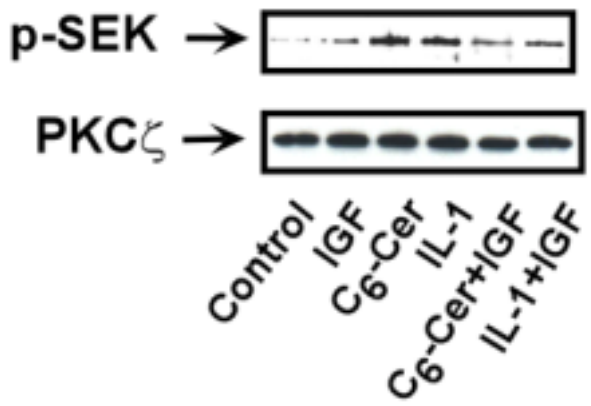

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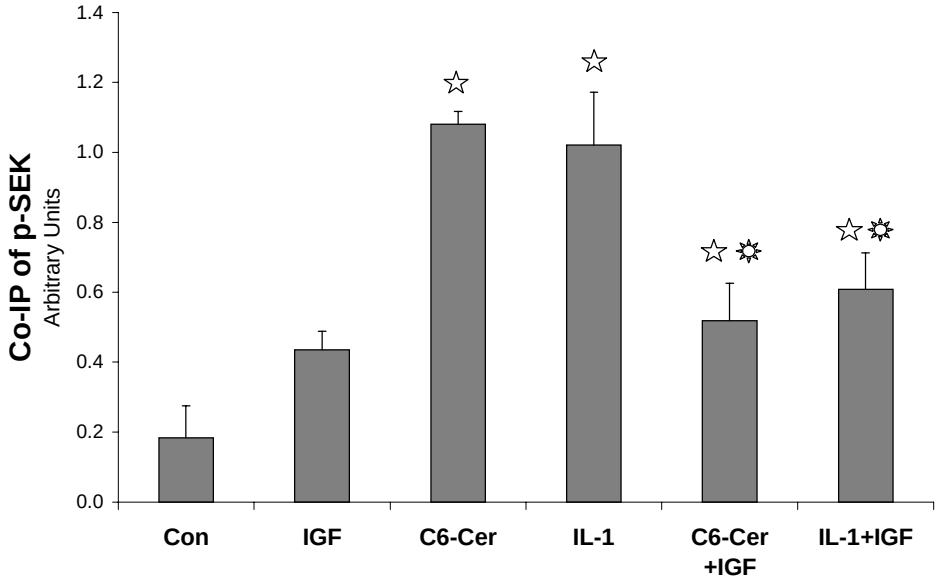
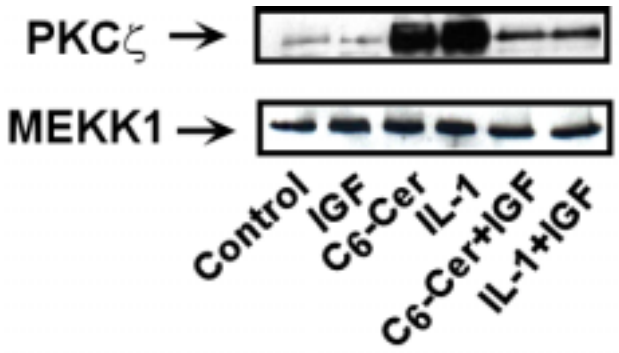


Figure 18. Ceramide induces PKCζ association with p-SEK and MEKK1. HEK 293 cells were used to assess PKCζ interaction with bioactive phospho-SEK (p-SEK) (**18A and 18B**) as well as MEKK1 (**18C and 18D**). Cells were treated with C_6-Ceramide (C_6-Cer, 1 μM), IL-1 (20 ng/ml) and/or IGF-1 (50 ng/ml) for 5-10 minutes. To assess PKCζ•p-SEK interactions, the lysed cells were immunoprecipitated with the anti-PKCζ antibody and immunoblotted with an antibody that detects phosphorylated SEK. Positive and negative controls included whole cell lysates and cell-free samples, respectively (data not shown). Both C_6-Ceramide and IL-1 treatment led to a significant interaction between PKCζ and p-SEK, which was reduced by co-treatment with IGF-1. **18A** depicts representative Western blots of three such experiments each. **18B** depicts the quantification of p-SEK and PKCζ protein levels in the complex, respectively. Mean ± Standard Error; n= 3; ☆ , significantly different from vehicle control; $p<0.01$, unpaired t-Test. ✡, Combination treatment is significantly different from individual treatment alone; $p < 0.01$, unpaired t-Test.

C



D

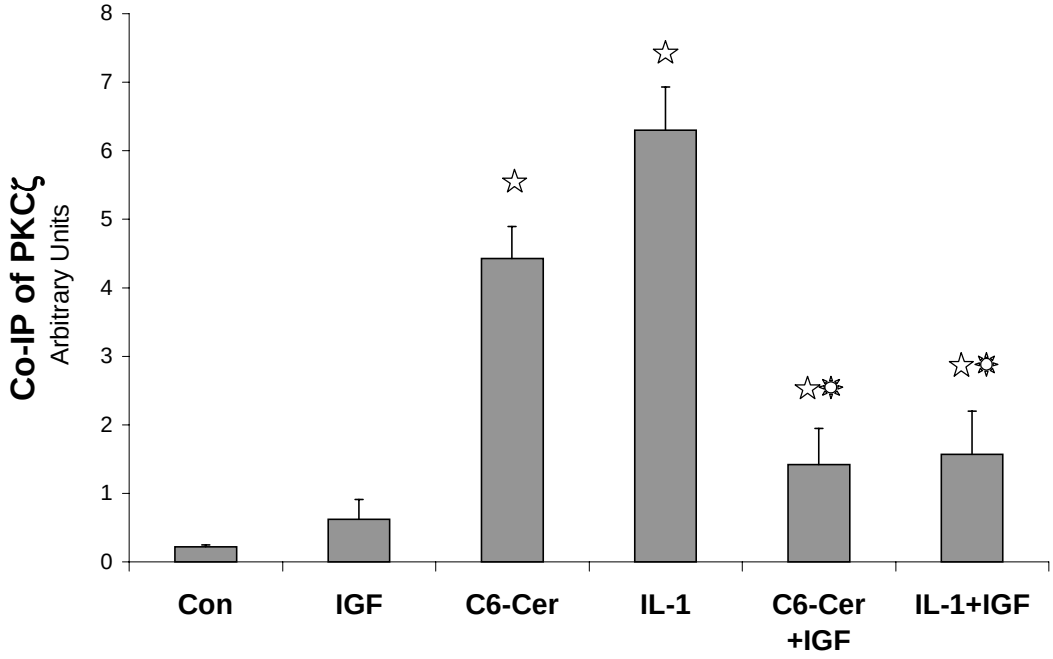


Figure 18. Ceramide induces PKCζ association with p-SEK and MEKK1. Cells were treated with C_6-Ceramide (C_6-Cer, 1 μM), IL-1 (20 ng/ml) and/or IGF-1 (50 ng/ml) for 5-10 minutes. To assess PKCζ•MEKK1 interactions, the lysed cells were immunoprecipitated with an anti-MEKK1 antibody and immunoblotted with the anti-PKCζ antibody. Reprobing the blots with the appropriate antibody (anti-PKCζ for 18A and anti-MEKK1 for 18C) assessed equal loading. Positive and negative controls included whole cell lysates and cell-free samples, respectively (data not shown). Both C_6-Ceramide and IL-1 treatment led to a significant interaction between PKCζ and MEKK1, which was reduced by co-treatment with IGF-1. **18C** depicts representative Western blots of three such experiments each. **18D** depicts the quantification of PKCζ protein levels in the complex, respectively. Mean ± Standard Error; n= 3; ☆ , significantly different from vehicle control; $p<0.01$, unpaired t-Test. ✡, Combination treatment is significantly different from individual treatment alone; $p < 0.01$, unpaired t-Test.

To define further the upstream SAPK signaling elements regulated by ceramide-activated PKCζ, we also investigated whether ceramide induces an association between PKCζ and MEKK1. As shown in **Figure 18C** and **18D**, we observed a strong association of PKCζ with MEKK1 in response to ceramide or IL-1 treatment. Again, IGF-1 reduced both C_6-ceramide- and IL-1-induced PKCζ•MEKK1 interactions. These results strongly suggest that stimulation by ceramide leading to SAPK activation is a result of stimulated PKCζ associating with MEKK1 as well as SEK.

Ceramide-Induced Growth Arrest is Dependent on PKCζ.

The critical role of PKCζ in ceramide- and IL-1-mediated inhibition of cell growth was assessed in HEK 293 cells transiently transfected with either Wild-Type (WT) or Constitutively-Active (CA) PKCζ. Cell cycle arrest was evaluated by Proliferating Cell Nuclear Antigen (PCNA) expression (**Figure 19**). PCNA expression is used as a marker of cells entering the cell cycle at early G1 and S phases. In cells transfected with the WT PKCζ construct, both IL-1 and its second messenger, ceramide, significantly reduced IGF-1-induced PCNA expression. This inhibitory effect of C_6-ceramide on HEK 293 cell growth does not appear to be caused by necrosis as C_6-ceramide, at concentrations up to 100 μM, did not induce LDH release (data not shown). In contrast to C_6-ceramide, the inactive cell-permeable ceramide analogue, dihydro-C_6-ceramide, did not have an inhibitory effect upon either basal or IGF-1-induced PCNA expression.

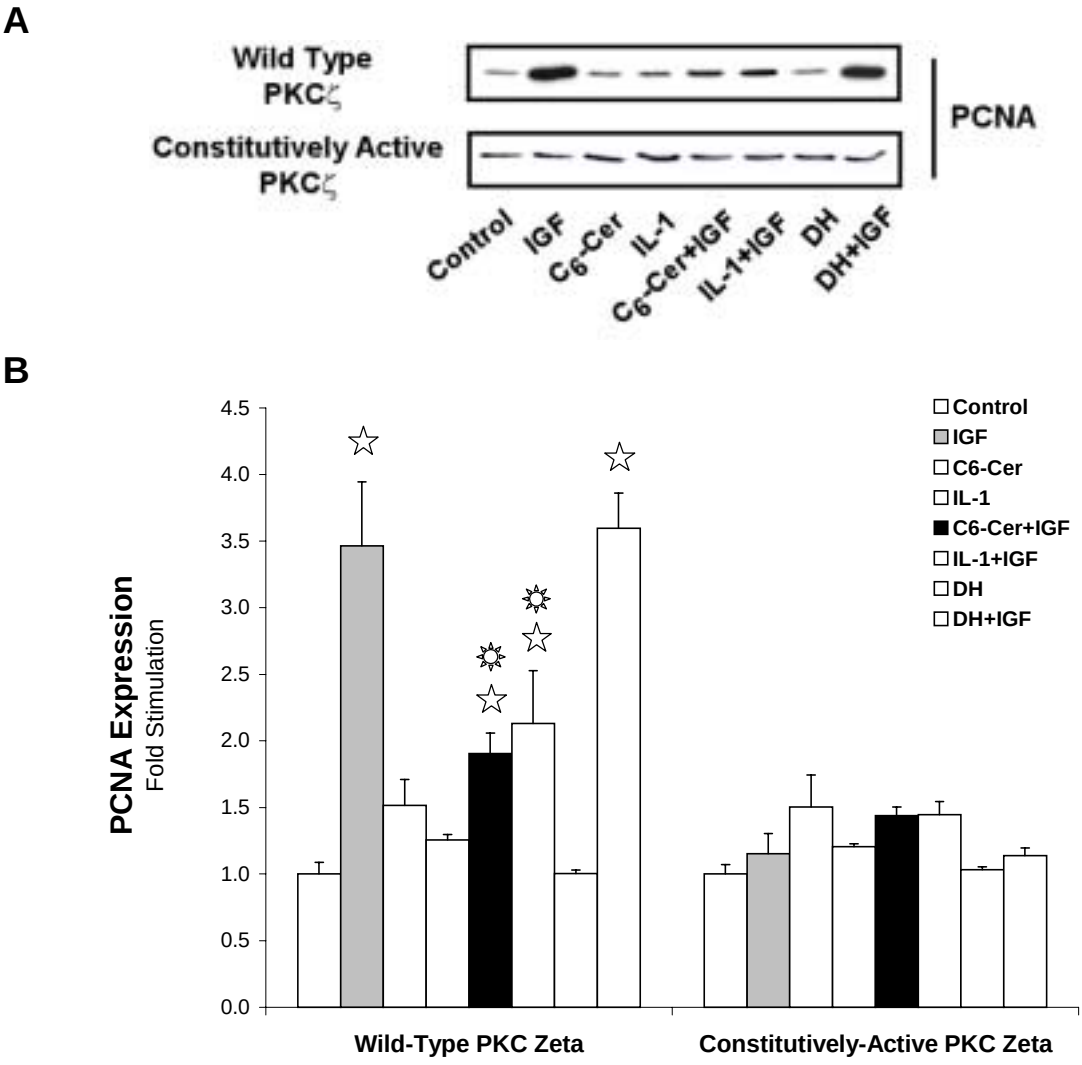


Figure 19. Ceramide-induced cell cycle arrest is dependent upon PKCζ. HEK 293 cells were transfected with either wild type or constitutively-active PKCζ constructs. After transfection, cells were treated with ceramide (1 μM), IL-1 (20 ng/ml) or dihydro-C_6-ceramide (DH, 1 μM) with or without IGF-1 (50 ng/ml) for 18 hours. Western analysis was performed on the cell lysates to assess PCNA expression. Both C_6-ceramide and IL-1 treatment significantly reduced IGF-induced PCNA expression in wild-type transfected cells. The specificity of the actions of ceramide was confirmed by the lack of an effect with dihydro-C_6-ceramide. The constitutively-active PKCζ expressing cells mimicked the actions of C_6-ceramide and IL-1 to induce cell cycle arrest in either wild-type, mock transfected or non-transfected cells. **19A** depicts representative Western blots. **19B** graphically illustrates the quantification of the PCNA bands. Mean ± Standard Error; n=4. ☆ significantly different from vehicle control; $p < 0.01$, unpaired Mann-Whitney test. ☼, significantly different from IGF-stimulated condition; $p < 0.05$, unpaired Mann-Whitney test.

Additionally, the mitogenic effect of IGF-1 was reduced in cells over-expressing the constitutively-active PKCζ. Compared to wild-type PKCζ expressing cells, a similar pattern of PCNA expression was observed in non-transfected or mock-transfected cells (data not shown). Collectively, these results suggest that PKCζ may be necessary for IL-1 and/or ceramide-induced cell cycle arrest. Moreover, these data imply that PKCζ•SAPK complex formation is required for ceramide-induced growth arrest (**Figure 20**).

3.4 PKCζ and Akt1

Ceramide induces an association between Akt-1 and PKCζ in A7r5 cells.

Another important pathway in cell survival and proliferation is the PI_3K cascade. A well-characterized downstream effector of the PI_3K pathway is Akt-1. Recent studies have demonstrated an ability of ceramide to inhibit Akt-1 activity [Salinas *et al*., 2000; Schubert *et al*., 2000; Summers *et al*., 1998; Zhou *et al*., 1998]. However, the precise mechanism by which Akt-1 inhibition by ceramide is achieved is not clear. One possible mechanism for ceramide inhibition of Akt-1 is through PKCζ. Therefore, we assessed the ability of ceramide to induce an association of Akt-1 with PKCζ in A7r5 cells. As shown in **Figure 21**, we observed a strong association of PKCζ, but not PKCε, with Akt-1 in response to C_6-ceramide, but not to DH-C_6-ceramide, treatment. In contrast, IGF-1 treatment not only failed to enhance this association between Akt-1 and PKCζ, but also significantly inhibited the effect of ceramide pre-treatment. Therefore, we concluded that PKCζ was a potential component in the ceramide-mediated

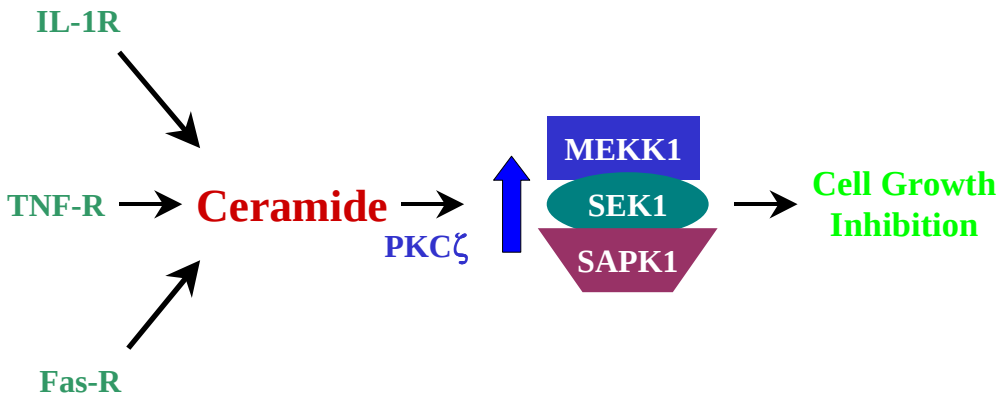
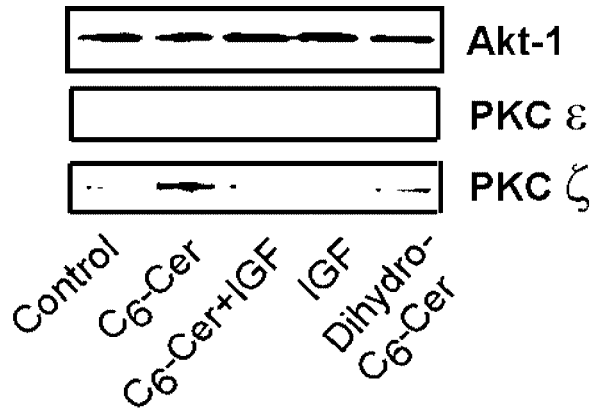


Figure 20. Mechanism by which ceramide activates the SAPK cascade.

A



B

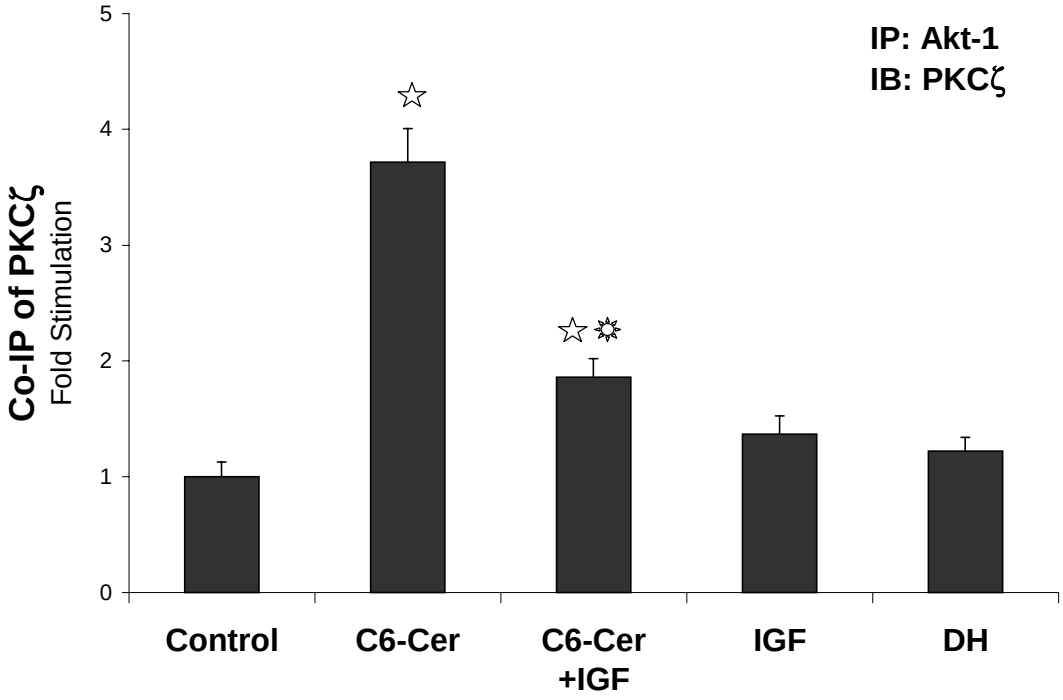


Figure 21. Ceramide enhances the interaction of PKCζ, but not PKCε, with Akt-1 in A7r5 cells. Protein interactions between select PKC isoforms and Akt-1 were assessed by Western analyses utilizing anti-PKCζ or -PKCε antibodies on Akt-1 immunoprecipitates from A7r5 cells. The cells were pre-treated with either C_6-ceramide or DH-C_6-ceramide for 1 hour and then treated with or without vehicle control (0.01% DMSO) or IGF-1 (50 ng/ml) for 5 minutes. C_6-Ceramide enhanced interactions of PKCζ, but not PKCε, with the Akt-1 protein. **21A** depicts representative Western blots. **21B** graphically illustrates the quantification of the PKC bands. Mean ± Standard Error; n=5; ☆, significantly different from vehicle control; $p < 0.001$, unpaired Mann-Whitney test. ✡, Combination treatment is significantly different from individual treatment alone; $p < 0.005$, unpaired Mann-Whitney test.

inhibition of Akt-1.

PKCζ is a necessary component for ceramide-induced inhibition of phospho-Akt-1 expression in A7r5 cells.

We have demonstrated that ceramide treatment increased association of PKCζ with Akt-1. Therefore, we assessed the necessity of PKCζ in ceramide-induced inhibition of bioactive, phosphorylated Akt-1 (p-Akt-1). For these experiments, we utilized wild-type and dominant-negative PKCζ constructs (previously described above). As shown in **Figure 22**, A7r5 cells over-expressing wild-type PKCζ treated with PDGF, resulting in significantly up-regulated p-Akt expression, consistent with growth factor stimulation of the PI_3K pathway. However, pretreatment with either C_2- or C_6-ceramide significantly decreased PDGF-stimulated p-Akt expression to basal levels. Conversely, in cells transfected with dominant-negative PKCζ, pretreatment with either C_2- or C_6-ceramide had no inhibitory effect on PDGF-stimulated p-Akt expression. In all cases, DH-C_6-ceramide, the inactive ceramide analogue, had no effect on p-Akt expression. Mock-transfection gave results similar to those from wild-type PKCζ constructs (data not shown). These experiments suggest that PKCζ is a necessary component for ceramide-induced inhibition of Akt-1 activity.

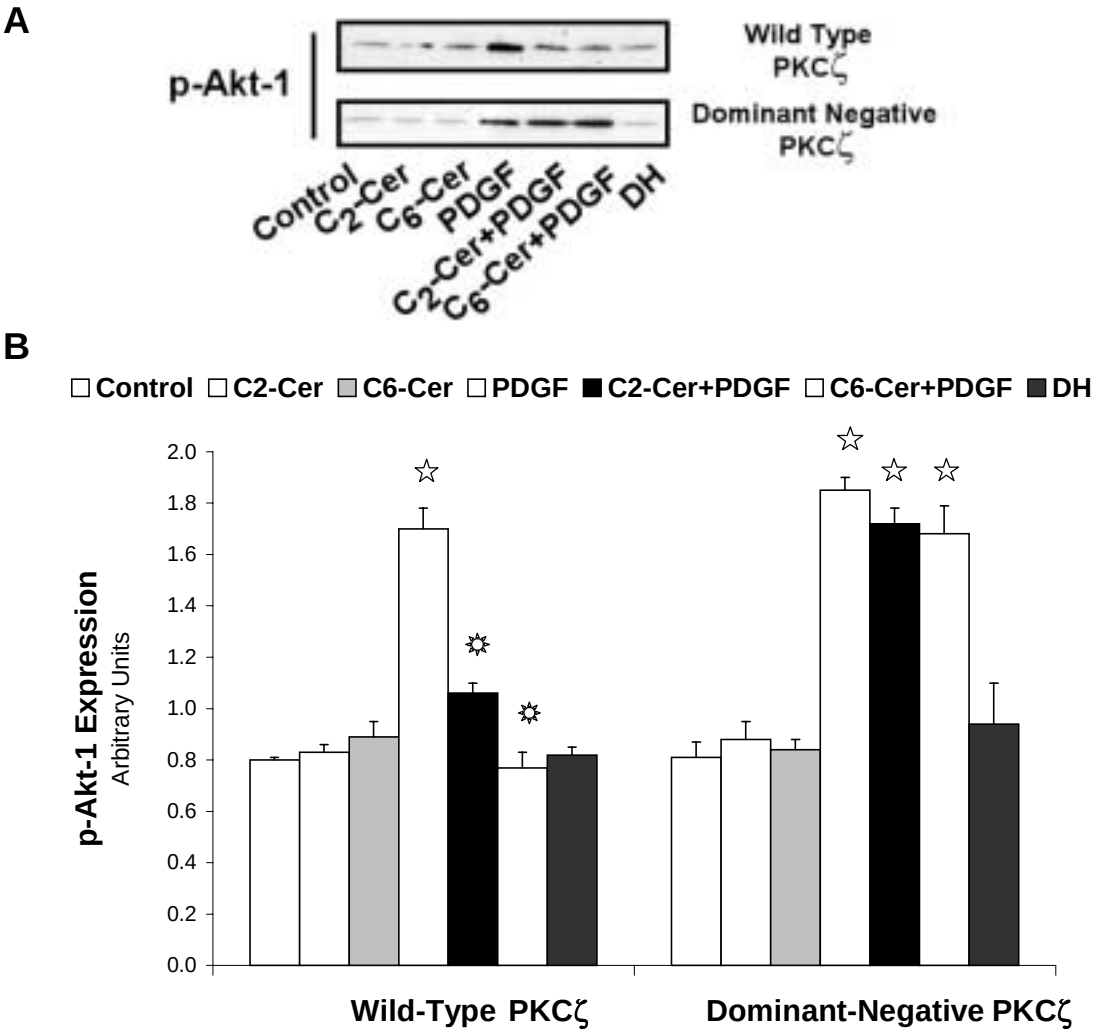


Figure 22. PKCζ is necessary for ceramide-mediated inhibition of PDGF-stimulated phospho-Akt-1. A7r5 cells were transfected with either wild type or dominant-negative PKCζ constructs. After transfection, cells were pre-treated with C_2- or C_6-ceramide (1 μM) or DH-C_6-ceramide (1 μM) for 1 hour and then treated with or without PDGF (10 ng/ml) for 5 minutes. Western analysis was performed on the cell lysates to assess p-Akt-1 expression. Both C_2- and C_6-ceramide, but not DH, pretreatment significantly reduced PDGF-induced p-Akt-1 expression in wild-type transfected cells. However, in cells over-expressing dominant-negative PKCζ, the ability of ceramide to inhibit PDGF-stimulated p-Akt-1 was abrogated. These results suggest that PKCζ is necessary for ceramide inhibition of p-Akt-1. Mock transfection produced similar patterns of p-Akt-1 expression (data not shown). **22A** depicts a representative Western blot. **22B** graphically illustrates the quantification of the p-Akt-1 bands. Mean ± Standard Error; n=4. ☆, significantly different from vehicle control; $p < 0.002$, unpaired t-Test. ✡, significantly different from PDGF-stimulated condition; $p < 0.005$, unpaired t-Test.

Activation of PKCζ by ceramide may be partially dependent upon PI_3K in A7r5 cells.

As mentioned previously, Akt-1 is a well-established effector molecule of the PI_3K pathway. There is controversy in the literature concerning whether ceramide inhibition of Akt-1 is PI_3K-dependent. Several reports indicate that Akt-1 inhibition by ceramide is PI_3K independent [Chen *et al*., 1999; Meier *et al*., 1998]. By contrast, other laboratories observed ceramide inhibition of Akt-1 to be PI_3K-dependent [Zundel & Giaccia, 1998]. Having demonstrated the importance of PKCζ in Akt-1 inhibition, we utilized the known PI_3K antagonist LY294002 (LY) to assess the effect of PI_3K inhibition on PKCζ activity. As shown in **Figure 23**, both C_6-ceramide and IL-1, but not DH-C_6-ceramide, increase PKCζ activity, consistent with our previous studies. However, with LY pretreatment, PKCζ activity is significantly decreased after either ceramide or IL-1 treatment. Pretreatment with LY had no effect upon DH-C_6-ceramide treatment. Since PKCζ was not restored to basal levels with PI_3K inhibition, these studies suggest that PKCζ may be partially dependent upon the PI_3K pathway.

In related experiments, we utilized another well-known PI_3K antagonist, wortmannin (Wort). As shown in **Figure 24**, we again observed a significant increase in PKCζ activity with C_6-ceramide and IL-1 treatment, but not DH-C_6-ceramide. Similar to results obtained by LY pretreatment, pretreatment with wortmannin led to a significant decrease in PKCζ activity. Wortmannin had no effect upon DH treatment. However, like the LY experiments, treatment with wortmannin did not bring PKCζ activity to basal levels, suggesting that

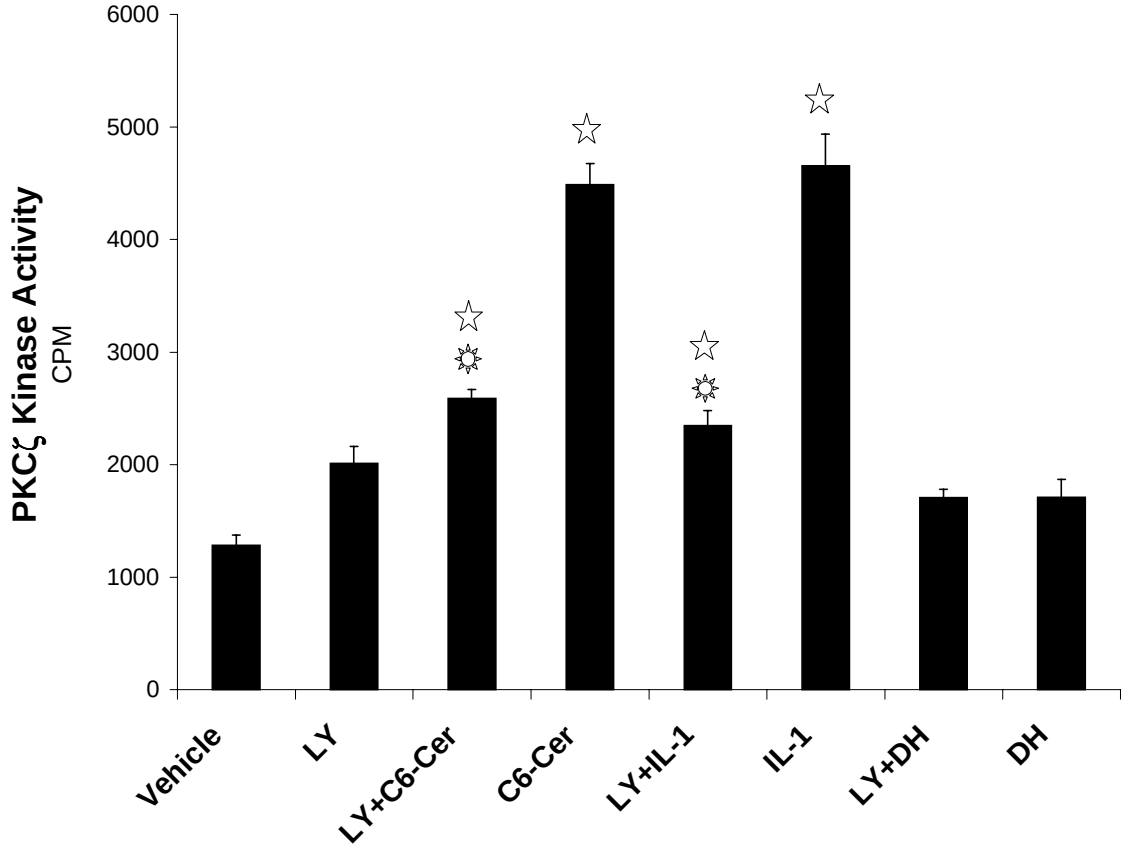


Figure 23. Inhibition of PI_3K by LY294002 decreases the ability of ceramide to stimulate PKCζ activity. PKC kinase activity assays were performed to assess the ability of IL-1 and C_6-ceramide to stimulate PKCζ after PI3K inhibition by LY294002 (LY). After pretreatment with LY (1 μM) for 30 minutes, ceramide (1 μM) and IL-1 (20 ng/ml), but not DH-C_6-ceramide (1 μM), were able to activate PKCζ, but to a significantly less extent than either ceramide or IL-1 alone. This figure represents the averaged counts per minute from three independent experiments. Mean ± Standard Error; n=3;☆ , significantly different from vehicle control; p< 0.01, unpaired t-Test. ✡ , Combination treatment is significantly different from individual treatment alone; p < 0.01, unpaired t-Test.

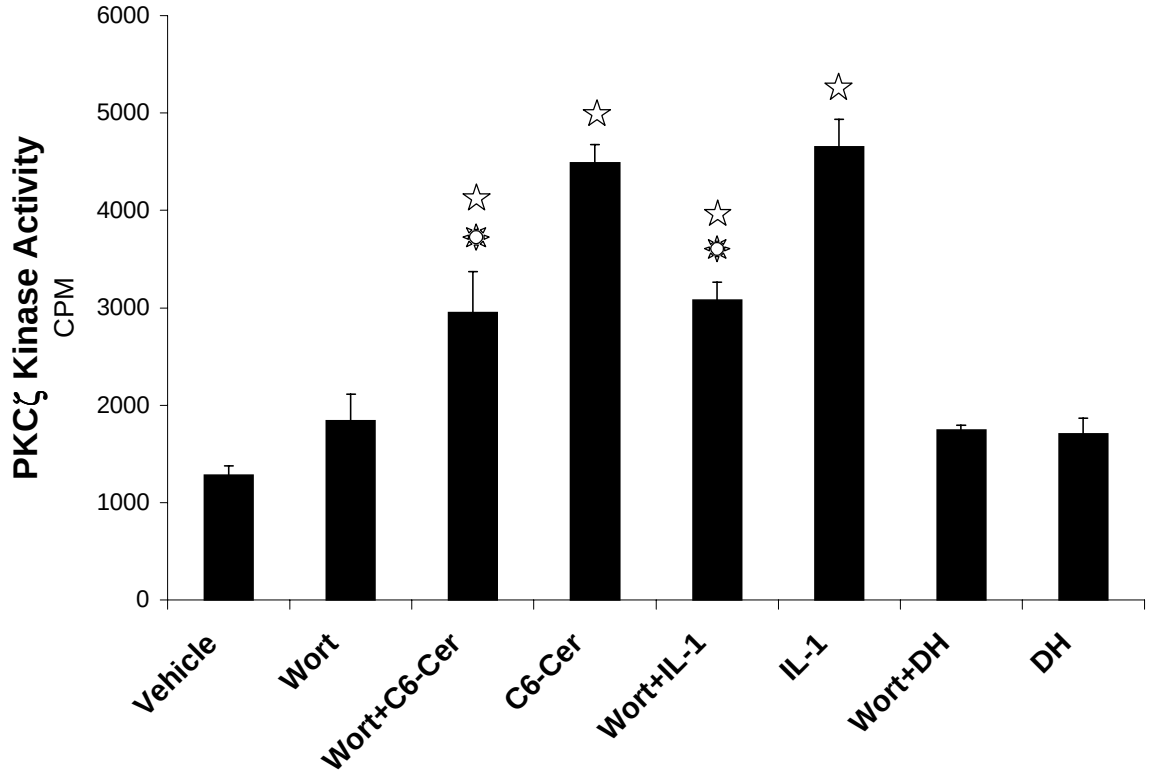


Figure 24. Inhibition of PI_3K by wortmannin decreases the ability of ceramide to stimulate PKCζ activity. PKC kinase activity assays were performed to assess the ability of IL-1 and C_6-ceramide to stimulate PKCζ after PI3K inhibition by Wortmannin (Wort). After pretreatment with Wort (100 nM) for 30 minutes, ceramide (1 μM) and IL-1 (20 ng/ml), but not DH-C_6-ceramide (1 μM), were able to activate PKCζ, but to a significantly less extent than either ceramide or IL-1 alone. This figure represents the averaged counts per minute from three independent experiments. Mean ± Standard Error; n=3; ☆, significantly different from vehicle control; $p < 0.01$, unpaired t-Test. ✡, Combination treatment is significantly different from individual treatment alone; $p < 0.01$, unpaired t-Test.

mechanisms other than PI_3K may be responsible for PKCζ activity. Collectively, both of these studies and their similarity in pattern suggest that PKCζ activity may be at least partially dependent upon PI_3K.

Since we demonstrated a potential role for PI_3K in PKCζ activity, we were interested in determining the role of PI_3K in ceramide-mediated inhibition of Akt-1. In order to do this, we are currently conducting PI_3K assays. In these PI_3K assays, we are investigating effects of ceramide upon PI_3K activity, as assessed by phosphorylation of exogenous phosphoinositide. With results from these experiments, we hope to define further mechanisms by which ceramide mediates its inhibitory effects upon Akt-1. The mechanism for inhibition of Akt1 by ceramide currently conceived is shown in **Figure 25**. Our experiments demonstrated not only that ceramide inhibits Akt1 activity, but that this inhibition depends upon PKCζ. Further experiments are necessary to determine the involvement of PI_3K in this system.

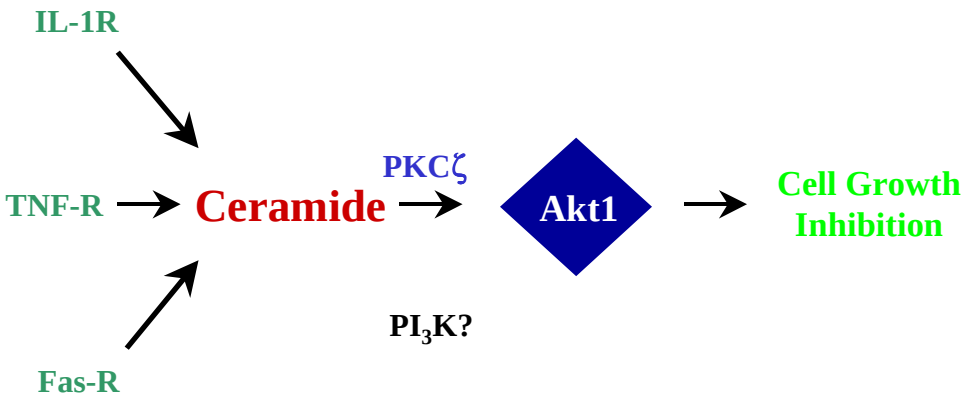


Figure 25. Proposed mechanism by which ceramide inhibits Akt1.

Chapter 4

DISCUSSION

These experiments have explored and characterized mechanisms by which the bioactive second messenger ceramide regulates growth arrest of activated vascular smooth muscle cells. First, we demonstrated that ceramide not only directly inhibits PKCε activity but also inhibits subsequent stimulation of the ERK cascade. Furthermore, we determined that ceramide inhibits complex formation of PKCε with upstream components of the ERK cascade, including Raf-1 and ERK. Second, we demonstrated that ceramide directly activates PKCζ, thereby up-regulating the SAPK cascade. Activation of the SAPK cascade leads to formation of signal complexes comprised of PKCζ and the upstream components of the SAPK cascade, MEKK1, SEK1 and SAPK. Lastly, we investigated the mechanism by which ceramide inhibits Akt1 activity. We determined that PKCζ is necessary to mediate this inhibition, but have not yet ascertained whether PI_3K or PDK1 is involved in this pathway. Collectively, we have demonstrated multiple mechanisms by which ceramide induces growth arrest. These mechanisms include direct regulation of kinases and subsequent formation of signal complexes.

4.1 Ceramide effects on proliferation and growth arrest.

Before we investigated the actions of ceramide at a mechanistic level, it

was essential to establish the ability of ceramide to curtail or inhibit the proliferative response of IGF-1 in HEK 293 cells. Our studies utilizing both PCNA expression and thymidine incorporation strongly support our hypothesis that ceramide induces cell cycle arrest in HEK 293 cells. These results agree with studies conducted in other laboratories investigating effects of ceramide upon proliferation [Fishbein *et al*., 1993; Jayadev *et al*., 1995; Westwick *et al*., 1995]. Although ceramide is well established in the literature to induce growth arrest or apoptosis, one report suggests that ceramide activates Raf-1, leading to activation of the ERK cascade [Yao *et al*., 1995]. This effect may be mediated by signaling through Raf-1 which could be converted to an apoptotic response in the presence of a small amount of BAD, a Bcl-2 family member [Basu *et al*., 1998]. Some studies suggest that the differences in ceramide pathway selectivity may be due to involvement of a particular isoform of sphingomyelinase, the enzyme that hydrolyses sphingomyelin to form ceramide [Adam *et al*., 1996; Adam-Klages *et al*., 1996; Wiegmann *et al*., 1994]. These studies suggest that stimuli which induce activation of acidic sphingomyelinases signal through the SAPK pathway, leading to apoptosis. Alternatively, stimuli that induce activation of neutral sphingomyelinases may transduce signals through the ERK cascade. These intriguing suggestions deserve further exploration.

Although our results are in accord with other published reports indicating that ceramide induces growth arrest, our methodology for evaluating proliferation could be improved. As discussed previously, assessment of PCNA expression is a rather crude method to determine cell cycle progression. [^{3}H]-thymidine

incorporation is preferable to assess proliferation. However, this technique has its own limitations. For example, [^{3}H]-thymidine can be passively taken up by cells, thus giving a falsely high incorporation. Another more accurate method to assess cell cycle progression is fluorescence-activated cell sorting (FACS) analysis. FACS analysis can provide data about the distribution of a population of cells in the cell cycle. With this technique, one can actually "see" where the cells are in the cell cycle, rather than just rely on markers of cells undergoing proliferation.

We can conclude from our experiments and other reports published in the literature that ceramide is capable of inducing cell growth arrest. Assessment of cell phenotype reveals little about the complex regulation occurring within the cell. Our mechanistic studies demonstrated that ceramide-mediated growth arrest involves regulation of at least 3 important mammalian signaling cascades: the ERK, SAPK and PI_3K/Akt1 pathways. However, these three pathways do not constitute the only mechanisms by which ceramide induces growth arrest. Furthermore, the effects of ceramide may be cell-type specific.

4.2 PKCε and ERK

We have investigated the role that ceramide plays in the inhibition of the ERK cascade, a well-known pathway leading to cellular proliferation. We observed that ceramide not only inhibits both immunoprecipitated and recombinant PKCε activity, but also negatively modulates cellular responses by antagonizing the protein•protein interaction of kinases involved in mitogenic

signaling pathways, such as ERK. This inability of PKCε to form a signaling complex with the upstream components of the ERK cascade results in decreased ERK activation.

The mechanism by which growth factors and their receptors regulate assembly of kinase signaling complexes between PKCs and elements of the ERK cascade is still unclear. The observation that ceramide-induced cell growth arrest, via inhibition of the ERK cascade, as a consequence of inactivated PKCε, suggests the critical role of PKCε in mitogenesis. Supporting our observations, some laboratories have shown that down-regulation of PKCε inhibits G1/S transition in vascular smooth muscle cells, an event consistent with IL-1-induced growth arrest [Mangoura & Dawson, 1993; Sasaguri *et al*., 1993]. Other studies have demonstrated that over-expression of PKCε induces tumorigenicity in fibroblasts [Cacace *et al*., 1993; Mischak *et al*., 1993] and enhanced nerve growth factor-induced phosphorylation of ERK in PC-12 pheochromocytoma cells [Hundle *et al*., 1995]. In addition, the fact that Raf-1 is activated by PKCε suggests that PKCε may directly phosphorylate Raf-1. Supporting this hypothesis, the Ueffing laboratory [Ueffing *et al*., 1997] demonstrated that PKCε and Raf-1 co-immunoprecipitate from PKCε transformed NIH-3T3 cells, indicating that PKCε may activate Raf-1 through direct protein•protein interactions. Collectively, the role of ceramide to limit selectively interaction between PKCε and Raf-1•ERK may illustrate one mechanism by which a pro-inflammatory response can be maintained in the absence of cell growth.

The concept of signaling complexes, in which the assembly and interactions of multiple kinases in large-scale aggregates determine the specificity and selectivity of cellular responses, is gaining widespread acceptance. Furthermore, it has been shown that some complexes of proteins are held together by scaffolding proteins. The role of scaffolding and/or adapter proteins, such as MP-1 (MEK partner-1) RICKs (Receptor for Inactivated C-Kinase), RACKs (Receptor for Activated C-Kinase), KSR (Kinase Suppressor of Ras) and 14-3-3 proteins, in assembling these signaling aggregates for the ERK cascade is only recently being appreciated [Elion, 1998; Schaeffer *et al*., 1998; Mochly-Rosen, 1995; Stewart *et al*., 1999; Whitmarsh *et al*., 1998]. These scaffolding proteins have a high degree of specificity and selectivity for binding proteins. Scaffolding proteins may serve either to promote interaction between proteins or to sequester proteins, thereby preventing interaction with other proteins, or both. In addition, scaffolding proteins may regulate subcellular localization of certain proteins, thereby separating them from cellular targets [Mochly-Rosen, 1995]. The importance of these proteins in ERK signaling is evident as yeast have similar scaffolding homologues for the MAPK cascade [Choi *et al*., 1994; Marcus *et al*., 1994; Printen & Sprague, 1994]. Although we did not investigate these scaffolding/adapter proteins in our studies, it might be of interest to assess the effects of ceramide on these coordinating proteins.

It is clear from our studies that ceramide inhibits PKCε activity and subsequent interaction with upstream components of the ERK cascade. However, other interpretations of our results merit exploration. Interactions

between PKC and the upstream components of the ERK cascade are based solely on co-immunoprecipitation studies. To show definitively direct protein•protein binding, we could utilize yeast 2-hybrid methodology. Alternatively, we could assess protein interactions using surface plasmon resonance. This technology allows for real-time assessment of protein-protein interactions for both association and dissociation. In addition, information on binding affinity, such as K_A and K_D, can be obtained from surface plasmon resonance technology. Therefore, in addition to assessing protein•protein interaction, we could also obtain insight into the strength of the interaction.

Questions remain regarding protein•protein interactions of the signaling complexes. Based upon co-immunoprecipitation studies, we have shown that PKCε interacts with upstream components of the ERK cascade. However, we have not shown that the signaling complexes are comprised of interactions at multiple sites. Multiple sites of interaction are possible since these proteins are held in close proximity to each other. Further studies could assess if PKCε interacts simultaneously with multiple members of the MAPK cascade.

Another area that could be further explored is the direct effect of ceramide upon PKCε. In our studies, we show, based upon transfections with dominant negative mutants, that a particular protein, PKCε, is necessary for activation of the ERK cascade. Although these data are highly suggestive that PKCε has a critical role in ERK activation, there remains the possibility of a component upstream of PKCε that may be more significant for ceramide-mediated inhibition of ERK signaling. One possibility is that some other target of ceramide, such as

CAPP, may mediate inhibition of the ERK pathway, and subsequent proliferation. To assess the contribution of CAPP, for example, we could perform experiments using known PP2A antagonists, such as okadeic acid.

Data obtained using the recombinant PKC protein yielded the strongest proof for direct ceramide inactivation of PKCε. However, even this *in vitro* assay may include a potential alternative site for ceramide to interact. The *in vitro* kinase assay includes phosphatidylserine (PS), a necessary cofactor for PKC activation. Therefore, we cannot exclude absolutely the possibility that ceramide may somehow interfere with PS binding to PKCε. However, the notion that ceramide may inhibit PKCε actions via inhibition of PS is unlikely, since we do not observe inhibition of PKCζ activity under similar conditions. In order to demonstrate absolutely direct inhibition by ceramide of PKCε, we would have to ascertain first the putative binding site(s) for ceramide. We would then have to generate site-directed mutants with deletions at the putative binding site(s). These experiments are currently being developed targeting the C1 cysteine rich lipid-binding domain of PKC.

In support of the rationale for ceramide directly binding to PKCε, previous studies in our laboratory showed that ether-linked diglyceride species competitively bound to the DAG binding site on PKCδ and ε without activating the kinase [Mandal *et al*., 1997]. As ceramide structurally resembles DAG, it is possible that ceramide competes against DAG for the putative DAG binding site. Ceramide could also bind to a secondary ceramide-binding site, rendering the PKCε insensitive to activation by DAG. Alternatively, when ceramide is bound to

PKCε, it may hinder PKCε from interacting with other proteins, such as Raf-1 and ERK. Whether ceramide directly competes with DAG for its putative C1-lipid-binding motif within PKCε is presently unclear. In fact, a radioiodinated photoaffinity-labeled ceramide analogue was unable to interact directly with immunoprecipitated non-activated PKCε [Huwiler *et al*., 1998]. Regardless of mechanism, our observation of ceramide-induced inactivation of immunoprecipitated and recombinant PKCε is supported by previous studies demonstrating that ceramide treatment induced translocation of PKCδ and PKCε from plasma membrane to cytosol [Jones & Murray, 1995; Sawai *et al*., 1997], an event consistent with inactivation. Ceramide has also been shown to inhibit PKCα activity [Lee *et al*., 1996], perhaps in a mechanism similar to PKCε inactivation by ceramide.

Our data indicate that one mechanism by which ceramide decreases ERK activity is via direct inhibition of PKCε and subsequent inability to form a signaling complex with Raf-1 and ERK. Other studies have postulated alternative mechanisms by which ceramide regulates the Raf-1•ERK cascade. Ceramide has been shown to bind to c-Raf [Pfeilschifter & Huwiler, 1998] as well as to KSR [Yao *et al*., 1995]. Ceramide binding to Raf-1 leads to sequestration of Raf-1 into inactive Ras•Raf-1 complexes [Muller *et al*., 1998]. Moreover, KSR has been shown to bind and functionally inactivate MEK1 [Denouel-Galy *et al*.; 1998; Yu *et al*., 1998]. Although ceramide may interact with upstream components of the ERK cascade, the end result appears to be the same. All these studies are consistent with decreased ERK activity. Finally, as mentioned above, a recent

study by the Kolesnick laboratory revealed that downstream targets, such as BAD, convert the normally pro-mitogenic ERK cascade into a ceramide-dependent pro-apoptotic signal pathway [Basu *et al*., 1998]. Thus, ceramide may regulate several mechanisms to inhibit ERK-mediated proliferation.

This novel role of ceramide to regulate protein•protein interactions, including PKCε•Raf-1•ERK interactions, is an attractive hypothesis by which inflammatory cytokine-induced ceramide formation may inhibit cellular proliferation.

4.3 PKCζ and SAPK

Another pathway we investigated was the SAPK cascade. As mentioned previously, this cascade is well-established for leading to growth arrest, differentiation and/or apoptosis. We were especially interested in this pathway, since in the literature ceramide has been demonstrated to activate this pathway [Coroneos *et al*., 1996; Westwick *et al*.; 1995; Verheij *et al*., 1996].

In our studies, we observed that ceramide not only activates both immunoprecipitated and recombinant PKCζ activity, but also positively modulates cellular responses by enhancing the protein•protein interaction of kinases involved in the SAPK pathway. This ability of PKCζ to form a signaling complex with the upstream components of the SAPK cascade results in increased SAPK activation. We elucidated a novel mechanism by which the sphingolipid metabolite, ceramide, can regulate protein-protein interactions between PKCζ and elements of the SAPK cascade, culminating in cell cycle arrest.

We demonstrated that ceramide selectively augments a signal complex formation of PKCζ with MEKK1, SEK and SAPK. As discussed with the ERK cascade, there are scaffolding/adapter proteins that may assist kinase complex formation for the SAPK/JNK pathway. The scaffolding proteins that may be critical in holding these complexes together include 14-3-3, JIP (Jun-Interacting Protein), RICKs and RACKs, to name a few [Fan *et al*., 2000; Harding *et al*., 2000; Kelkar *et al*., 2000; Mochly-Rosen, 1995]. The importance of these proteins is underscored by the fact that they are conserved from yeast to mammals [Choi *et al*., 1994; Marcus *et al*., 1994; Printen & Sprague, 1994]. We did not explore involvement of scaffold proteins in our experiments, but it would be of interest to determine which scaffold/adapter proteins are involved in our system.

Again, we could question the validity of our conclusions, primarily based on the fact that co-immunoprecipitation experiments formed the basis for our observations. We previously discussed some concerns and criticisms of our methodologies in the PKCε and ERK section. Those same considerations apply for PKCζ and SAPK as well. They include the direct binding and regulation of PKC by ceramide. Another concern is that our results may be artifacts of hydrophobic interactions arising from ceramide treatment. However, our lysis buffers contained the non-ionic detergent 0.2% NP-40. The fact that our experiments were conducted in the presence of non-ionic detergent lends credence to our conclusions that direct protein-protein interactions are occurring as a consequence of ceramide activation of PKCζ and, presumably, not by

ceramide promoting hydrophobic associations of these proteins at the membrane. Based on this evidence, as well as the recombinant PKCζ studies, we suggest that direct interaction and activation of PKCζ by ceramide leads to recruitment and activation of upstream elements in the SAPK cascade, resulting in cell cycle arrest.

Although our studies show a direct link between ceramide and the SAPK upstream components, there may exist other possible mechanisms of SAPK activation that we did not explore. In fact, other studies have postulated alternative mechanisms by which ceramide regulates the SAPK cascade. Ceramide has been shown to activate small molecular weight G-proteins that may couple inflammatory cytokine receptors with the SAPK cascade [Brenner *et al*., 1997; Coso *et al*., 1995]. Specifically, ceramide stimulates Rac-1, as well as Vav, a guanine nucleotide exchange factor for Rac [Esteve *et al*., 1998]. Another SAPK regulatory mechanism may involve ceramide activation of PP1 and PP2A phosphatases [Chalfant *et al*., 1999]. Even though over-expression of a dual-specificity threonine/tyrosine phosphatase, M3/6, diminishes ceramide-activated SAPK [Smith *et al*., 1997], other studies demonstrate that ceramide stimulates SAPK under conditions where ceramide also activates protein phosphatases [Coroneos *et al*., 1996; Westwick *et al*., 1995]. The role of ceramide-activated phosphatases in modulating ceramide-dependent PKCζ•SAPK interactions is of interest. Thus, ceramide may regulate several mechanisms mediating SAPK-induced cell cycle arrest.

The role of ceramide binding to, and activating, PKCζ remains controversial. Our studies, using both immunoprecipitated and recombinant human PKCζ, demonstrate that ceramide, but not dihydroceramide, directly induces PKCζ bioactivity. Supporting our findings, ceramide has been shown to bind to PKCζ as determined by kinetic analyses and *in vitro* phosphorylation studies [Bieberich *et al*., 2000; Muller *et al*., 1995]. In contrast, a radioiodinated photoaffinity-labeled ceramide analogue was unable to interact directly with immunoprecipitated PKCζ [Huwiler *et al*., 1998]. These apparent contradictions in the literature may be due to structural differences in the ceramide analogues. Alternatively, the ability of ceramide to interact with the cysteine-rich lipid-binding domain (CRD) of immunoprecipitated PKCζ could be altered by co-immunoprecipitating proteins, such as Par-4, that also interact with this domain [van Blitterswijk, 1998]. It has been proposed that the single CRD of PKCζ may interact with ceramide, but not with DAG [Coroneos *et al*., 1996; Hurley *et al*., 1997]. This may be due to the lack of a second CRD, which is observed in conventional and novel PKC classes, or to subtle differences in the loop structure of the PKCζ CRD that respond to the unique polar regions of ceramide [van Blitterswijk, 1998]. Although DAG does not directly activate PKCζ, it is suggested that DAG selectively inhibit PKCζ activity by antagonizing ceramide binding at the CRD. The fatty acyl groups of DAG may block this putative ceramide-binding domain in a fashion analogous to arachidonic acid blocking ceramide binding to PKCζ [Muller *et al*., 1995].

It is well established in the literature that PKCs are lipid-activated proteins. In fact, PKCs are one of the prototypes for lipid activated proteins. All PKCs are activated by PS, and many isoforms are activated by DAG. Therefore, because PS and DAG lipids can bind to PKCs, is it likely that other lipids bind to it as well? It is not simply a leap of faith that ceramide may be binding to PKCs. Evidence supports binding of a sphingolipid to PKCs [Hannun *et al*., 1986; Khan *et al*., 1991; Merrill *et al*., 1986; Weiss *et al*., 1991]. These studies demonstrate competitive binding of sphingosine, as well as some derivatives, resulting in inhibition of PKC. Therefore, it seems likely that, if ceramide does indeed bind to PKCε and/or PKCζ, this binding will be competitive. Competitive inhibition experiments between ceramide and DAG for PKCε and PKCζ are currently ongoing in the laboratory.

In our studies, we observed that the actions of ceramide upon PKCζ•SAPK interactions were inhibited by IGF-1 treatment. This surprising observation has several explanations. IGF-1-generated DAG may compete with ceramide at the putative ceramide binding site on PKCζ [van Blitterswijk, 1998] or activate other PKC isotypes linked to mitogenesis. In addition, IGF-1 also stimulates PKCζ through a PI_3K-dependent mechanism [Liu *et al*., 1998]. This alternate mechanism for stimulating PKCζ may couple PKCζ to other Mitogen Activated Protein Kinases (MAPK), such as Extracellular Signal-Regulated Kinases (ERK), which are more closely linked to mitogenesis. The role of sphingolipid- and polyphosphoinositide-derived second messengers to regulate differentially PKCζ•MAPK signaling complexes is an attractive theory beyond the

focus of the current studies. Alternatively, ceramide may become phosphorylated by IGF-1 treatment, generating ceramide-1-phosphate, a pro-mitogenic lipid [Carpio *et al*., 1999]. Another possibility suggests that IGF-1 treatment induces activation of ceramidase, an enzyme that catalyzes de-acylation of ceramide to form the pro-mitogenic lipids, sphingosine or sphingosine-1-phosphate [Spiegel, 1999]. Regardless of mechanism, IGF-1 co-treatment reduces the ability of inflammatory cytokines or ceramides to up-regulate SAPK activity. However, this compensatory mechanism does not supercede the ability of ceramides to induce cell cycle arrest.

Our observation that ceramide-induced cell growth inhibition is a consequence of activated PKCζ coupling to elements of the SAPK cascade suggests a critical role for specific PKC•MAPK signaling complexes in cell cycle arrest. This is novel since PKCζ can be activated by both mitogenic and anti-mitogenic stimuli. Thus, the ability of stimulated PKCζ to interact with distinct MAPK signaling elements could explain the contradictory actions of PKCζ as a regulator of cell growth. For example, it was initially demonstrated that PKCζ is required for maturation of *Xenopus* oocytes and for DNA synthesis in fibroblasts [Berra *et al*., 1993]. Interactions between PKCζ and the pro-mitogenic ERK cascades have been suggested, as a dominant-negative mutant of PKCζ suppressed stimulation of MEK and ERK by TNFα [Berra *et al*., 1995]. However, recent studies suggest a growth inhibitory role for PKCζ. NIH 3T3 fibroblasts transfected with wild-type PKCζ are not tumorigenic [Montaner *et al*., 1995]. In fact, PKCζ has been reported to suppress neoplastic transformation of fibroblasts

mediated by the v-Raf oncogene [Kieser *et al*., 1996] and does not enhance Raf-1 activity, an upstream kinase in the ERK cascade *in vitro* [Schonwasser *et al*., 1998; Sozeri *et al*., 1992]. These observations offer an explanation for inhibition of ERK bioactivity by ceramide [Coroneos *et al*., 1996; Mandal *et al*., 1997; Muller *et al*., 1998; Westwick *et al*., 1995]. Thus, the ability of ceramide to couple preferentially activated PKCζ to upstream elements in the SAPK cascade, and not the ERK cascade, could provide one mechanism for inducing cell cycle growth arrest.

We have shown that ceramide induces cell cycle arrest via selective interactions of PKCζ with elements of the SAPK cascade. Moreover, these interactions are modulated by the ability of ceramide to bind to and directly activate PKCζ. The role of ceramide to induce selectively PKCζ•SAPK complex formation may illustrate one mechanism by which a pro-inflammatory response can lead to cell growth arrest. This critical role of ceramide to activate directly PKCζ and to regulate MEKK1•SEK•SAPK interaction, is a novel hypothesis by which inflammatory cytokine receptor-induced ceramide formation may limit cellular proliferation. The ability of ceramide to activate the SAPK pathway in combination with inhibition of the ERK cascade represents a potent means for inducing growth arrest in our experimental system.

4.4 PKCζ and Akt-1

The MAPK pathways have been well characterized with regard to ceramide. However, more recent studies have investigated the effects of

ceramide upon another well-known signaling pathway, the PI_3K/Akt1 pathway. Ceramide has been shown to inhibit Akt1 through undefined mechanisms [Salinas *et al*., 2000; Schubert *et al*., 2000; Summers *et al*., 1998; Zhou *et al*., 1998]. As we have previously shown that ceramide activates PKCζ and others have shown that PKCζ directly binds to and inactivates Akt1 [Konishi *et al*., 1994a; Konishi *et al*., 1994b; Doornbos *et al*., 1999], we hypothesized that ceramide inactivates Akt1 in a PKCζ-dependent mechanism.

Our experiments not only demonstrated ceramide inhibition of Akt1 activity, in agreement with other published studies, but also demonstrated the involvement of PKCζ. What we did not demonstrate, however, was whether ceramide inhibition of Akt1 is also mediated through PI_3K. Our studies suggest, but did not prove, that ceramide- and IL-1-induced PKCζ activity may be partially dependent on PI_3K, based on studies utilizing known PI_3K antagonists. Caution should be exerted in the interpretation of these data, since both PKCζ and Akt1 have been shown to be downstream of PI_3K [Liu *et al*., 1998; Nakanishi *et al*., 1993; Standaert *et al*., 1997]. Moreover, we have not as yet investigated the effects of ceramide upon the PI_3K pathway in both whole cell and cell-free assays.

Complicating the issue are reports demonstrating activation of Akt1 independent of PI_3K [Konishi *et al*., 1996; Konishi *et al*., 1997; Sable *et al*., 1997]. These studies show PI_3K-independent activation of Akt1 by stress signals. The following year, a report appeared refuting these studies [Shaw *et al*., 1998]. Clearly, more work needs to be done on this cascade to demonstrate the

mechanism linking PI_3K and Akt1. The link between PI_3K and Akt1 may be clarified by the recent finding of a kinase intermediate between PI_3K and Akt1, which is phosphoinositide (3,4,5) P_3-dependent 3-kinase 1 (PDK1). Reports show that PDK1 is able to activate Akt1 in a PI_3K-dependent manner [Alessi *et al*., 1997; Didichenko *et al*., 1996; Klippel *et al*., 1996; Stokoe *et al*., 1997]. In addition, studies revealed that PI_3K-activation of PKCζ is mediated by PDK1 [Chou *et al*., 1998; Dutil *et al*., 1998; Le Good *et al*., 1998]. It appears that PDK1 is able to bind directly to and activate both PKCζ and Akt1, independently of each other. How precisely PDK1 regulates Akt1 remains to be explored. However, a recent report in the literature demonstrates that ceramide does not affect PDK1 activity, which is thought to be constitutively active [Salinas *et al*., 2000]. These investigators suggested that ceramide treatment may decrease Akt1 activity by inhibiting translocation to the nucleus, independent of PDK1 inhibition.

Another mechanism for inhibition of Akt1 activity is through activation of a ceramide-activated protein phosphatase [Chen *et al*., 1999; Meier *et al*., 1998; Salinas *et al*., 2000]. However, other studies suggested that this mechanism may not be plausible, as treatment with okadeic acid, a phosphatase inhibitor, did not prevent *in vitro* phosphorylation of Akt1 [Summers *et al*., 1998; Zhou *et al*., 1998]. The Summers studies used a fairly high concentration (1 μM) of okadeic acid. At this concentration, nonspecific inhibition of other phosphatases may come into play, whereas the Zhou studies utilized a more PP2A-specific concentration of 100 μM. Perhaps ceramide inactivates Akt1 via both PKCζ- and

CAPP- dependent mechanisms. Further studies need to be performed to clarify this regulation.

Our studies have demonstrated involvement of PKCζ in ceramide-mediated inhibition of Akt1. Additional studies ongoing in the laboratory will elucidate the mechanisms by which ceramide inactivates Akt1. At the present time, we cannot exclude the possibility of other non-PKCζ-dependent mechanisms for ceramide inhibition of Akt1. Regardless of mechanism, it appears that Akt1 activity, and perhaps PI_3K, is indeed subject to ceramide regulation. Thus, ceramide may also inhibit vascular smooth muscle cell proliferation via a PKCζ-dependent inactivation of Akt1.

Chapter 5

CONCLUSIONS

Taken together, our studies demonstrate the ability of ceramide to regulate three independent signal transduction cascades. We demonstrated two mechanisms of inhibitory actions of ceramide, and one mechanism for stimulatory actions of ceramide. **Figure 26** illustrates these proposed mechanisms. The differential regulation of biochemical pathways by a lipid-derived second messenger represents a novel regulatory mechanism. However, a larger question remains. How useful is this information?

As discussed in the introduction, the maintenance of cellular homeostasis is a fine balance between proliferative and apoptotic stimuli. Two pathways that have traditionally been associated with proliferation, ERK and PI_3K/Akt1, are negatively modulated by ceramide. Whereas the SAPK pathway, which has been shown to be involved in growth arrest, differentiation and apoptosis, is positively modulated by ceramide. The result of inhibiting two stimulatory pathways and activating one growth-arresting pathway leads to a net phenotype of growth arrest. The fact that ceramide is capable of inhibiting proliferation without inducing significant necrotic or apoptotic cell death in differentiated tissues has significant implication for therapeutic application in the inhibition or limitation of chronic inflammatory disease.

Our laboratory has utilized these growth-arresting properties of ceramide

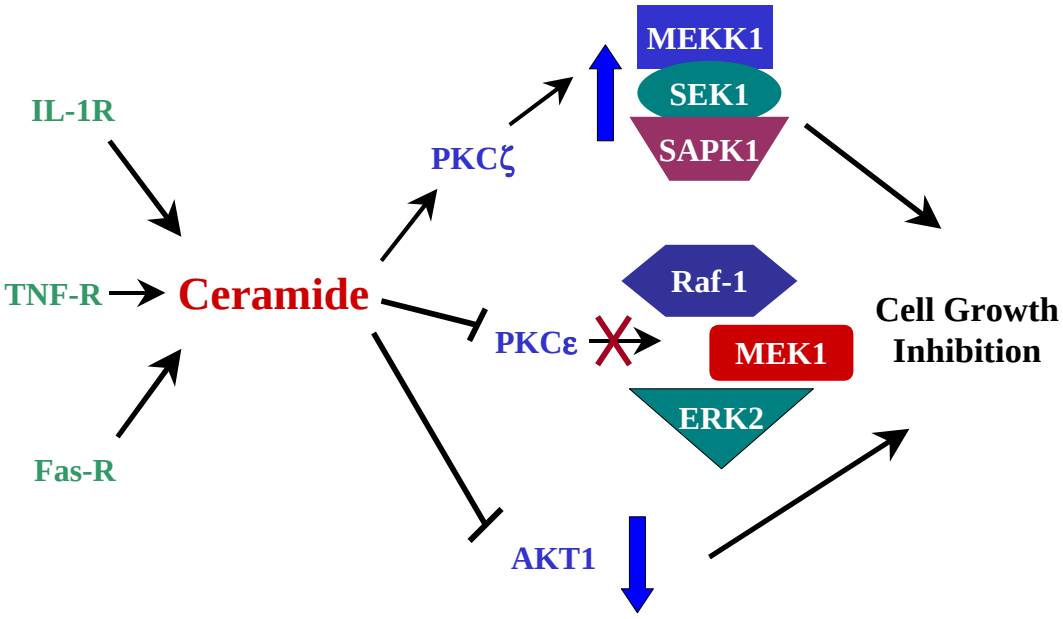


Figure 26. The mechanisms of ceramide-mediated growth arrest.

to inhibit neointimal hyperplasia in New Zealand white rabbits and, most recently, in swine. In control rabbit experiments, we applied ceramide at the site of injury with biomechanical force via the inflation of balloon catheters. Our hypothesis in these studies was that direct delivery of ceramide, a cell-permeable growth-arresting lipid, would limit neointimal hyperplasia after stretch injury. Our studies demonstrated that ceramide treatment significantly decreased the number of vascular smooth muscle cells entering the cell cycle without inducing apoptosis. Furthermore, we confirmed our *in vitro* mechanistic studies with the *in vivo* animal studies. Specifically, in the rabbit studies we found a decrease in both ERK and Akt1 activity. These rabbit experiments are currently being repeated in hyperlipidemic rabbit models as well as in iliac, renal and coronary vessels of swine.

Our preliminary data from these ongoing studies in the swine and hyperlipidemic rabbits demonstrate promising effects of ceramide application at the site of injury. Therefore, it appears that our *in vitro* studies correlate with whole animal studies, suggesting that ceramide does indeed halt proliferation in lesions or areas of injury and inflammation. Long-term therapeutic implications of the *in vitro* and *in vivo* studies include direct and localized application of ceramide on either stents or balloons.

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