Ceramide-induced Inhibition of Akt Is Mediated through Protein Kinase Cζ

IMPLICATIONS FOR GROWTH ARREST*

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We recently demonstrated that ceramide-coated balloon catheters limit vascular smooth muscle cell (VSMC) growth after stretch injury in vivo. In that study, inhibition of VSMC growth was correlated with a decrease in phosphorylation of the cell survival kinase Akt (protein kinase B). Utilizing cultured A7r5 VSMCs, we have now examined the mechanism by which ceramide inhibits Akt phosphorylation/activation. Our initial studies showed that ceramide-induced inhibition of Akt phosphorylation was not mediated through diminution in phosphoinositide 3-kinase activity. As we have previously demonstrated that protein kinase Cζ (PKCζ) is a target of ceramide, we proposed an alternative signaling mechanism by which ceramide induces inhibition of Akt through activation of PKCζ. We demonstrate that C6-ceramide (but not the inactive analog dihydro-C6-ceramide) induced PKCζ activity and also caused a selective increase in the association between Akt and PKCζ, without affecting PKCε, in A7r5 cells. In addition, the ability of ceramide to significantly decrease platelet-derived growth factor-induced Akt phosphorylation or cell proliferation was abrogated in A7r5 cells overexpressing a dominant-negative mutant of PKCζ. Taken together, these data suggest that ceramide-mediated activation of PKCζ leads to diminished Akt activation and consequent growth arrest in VSMCs. The therapeutic potential for ceramide to limit dysregulated VSMC growth has direct applicability to vascular diseases such as restenosis and atherosclerosis.

Ceramide is a sphingolipid-derived second messenger that has been implicated in growth arrest, differentiation, and/or apoptosis (1–3). Using human embryonic kidney cells, we have recently demonstrated that ceramide-induced growth arrest is associated with changes in protein kinase signaling cascades, including protein kinase C (PKC)1 and mitogen-activated protein kinase (4, 5). In particular, ceramide-mediated PKCζ activation leads to inhibition of cell growth (4). In addition, we (3) and others (6–10) have shown that ceramide inhibits cell growth through inhibition of the cell survival kinase Akt/protein kinase B in vivo and in vitro. However, the precise mechanism by which ceramide inhibits Akt activation has not been elucidated.

Multiple lines of evidence suggest that Akt is a downstream target of phosphoinositide 3-kinase (PI3K) in different cell types, including vascular smooth muscle cells (VSMCs) (11–16). Whether ceramide inhibits Akt activation through a PI3K-dependent mechanism is still a subject of controversy in the literature. Recent studies have shown that ceramide-induced inhibition of Akt occurs in a PI3K-dependent (6) and PI3K-independent (17, 18) manner. Of particular importance is the fact that PKCζ has been identified as a putative PI3K-independent regulator of Akt (19). Direct binding of PKCζ to Akt (20, 21) serves to negatively regulate Akt activation (19). As we (4) and others (22–24) have shown that PKCζ is a target of ceramide, it is now hypothesized that PKCζ may mediate ceramide-induced inhibition of Akt.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant platelet-derived growth factor (PDGF)-BB and insulin-like growth factor-1 (IGF-1) were from Invitrogen. Interleukin-1β (IL-1β) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti–PKCζ, anti–PKCε, anti–PKCαι, anti-p85α PI3K, anti-Akt-1, and anti-phospho-Akt (Ser473) primary antibodies were from either Santa Cruz Biotechnology (Santa Cruz, CA) or New England Biolabs Inc. (Beverly, MA). C6-, C2-, dihydro-C2-, and dihydro-C6-ceramide and l-α-phosphatidylinositol were from either Avanti Polar Lipids (Alabaster, AL) or BIOMOL Research Labs Inc. (Plymouth Meeting, PA). [γ-32P]ATP and [3H]Thymidine were from ICN Radiochemicals (Costa Mesa, CA). All other chemicals were purchased from either Fisher or Sigma.

Cell Culture and Treatments—Rat embryonic thoracic aorta smooth muscle-derived A7r5 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO2. After attainment of confluency (70–80%), the cells were incubated in serum-free DMEM containing 0.5% bovine serum albumin for 20–24 h. For experiments involving treatment with C6-, C2-, dihydro-C2-, or dihydro-C6-ceramide, the following protocol was used. A stock solution (200 μM) of ceramide derivative(s) was initially prepared in Me2SO. This was diluted 200-fold with serum-free DMEM containing 5% bovine serum albumin to obtain a working ceramide concentration of 1 μM (with constant stirring for 45 min at room temperature). To expose the cells to the ceramide derivative, the working ceramide solution was added to serum-free DMEM containing 0.5% bovine serum albumin to obtain the final concentration (1 or 10 μM) as indicated in the figure legends.

3H/Thymidine Incorporation Studies—Serum-starved A7r5 cells were pretreated without (control) or with ceramide derivative(s) for 1–2 h and then exposed to PDGF (10 ng/ml) for an additional 20 h in the presence of the above agents. During the last 4-h incubation period, the...
cells were labeled with [3H]thymidine (0.5 μCi/ml). After labeling, the cells were washed with ice-cold phosphate-buffered saline and then exposed to 10% trichloroacetic acid (3 × 10 min). After complete removal of the trichloroacetic acid, the acid-insoluble material was extracted with 0.1 N NaOH. The incorporation of [3H]thymidine into acid-insoluble DNA was determined with a liquid scintillation counter (15).

Western Blot Analyses—Both control and treated A7r5 cells were washed with ice-cold phosphate-buffered saline and exposed to lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EDTA, 1 mM Na3VO4, 0.2% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin). The cell lysates were then centrifuged at 14,000 × g for 10 min at 4 °C. The supernatants (40 μg of protein) from the cell lysates were subjected to 10% SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membranes (Hybond C, Amersham Biosciences, Inc., Uppsala, Sweden). The membranes were blocked in 5% nonfat milk in Tris-buffered saline for 1 h at room temperature. After incubation, the membranes were washed with Tris-buffered saline (3 × 10 min). The membranes were then incubated with the appropriate secondary antibody (1:5000 dilution) for 2 h at room temperature. After three more washes with Tris-buffered saline, the protein bands were detected by the enhanced chemiluminescence method and quantitated by laser densitometry (Molecular Dynamics, Inc., Sunnyvale, CA) (5, 15, 25). The protein concentration was determined by the Bradford method (BioRad, Hercules, CA).

To confirm the specificity of anti-PKCα primary antibody, the membranes were stripped (15) and reprobed with additional PKC isoform-specific antibodies as described previously (4, 5). A protein band corresponding to the molecular mass of PKCα was visualized by autoradiography and quantitated by laser densitometry. Equal loading of samples was verified by reprobing the blots with an anti-α-tubulin antibody.

Transient Transfection Studies with PKCα Constructs—A7r5 cells were transiently transfected with wild-type or dominant-negative mutant PKCα constructs (a generous gift from Dr. Shi-Ming Chen) (Qiagen Inc.) according to the manufacturer's instructions. Superfect reagent consists of non- lipid-activated dendrimer molecules, which assemble DNA into compact structures and optimize DNA entry into cells. The wild-type construct is full-length PKCα in a pcDNA3 expression vector. The dominant-negative mutant construct is a kinase-defective mutant that contains a point mutation in the catalytic domain. Briefly, A7r5 cells were transfected (75%–80%) with 1 μg of pcDNA3 (supplemented with 10% fetal bovine serum) along with a mixture of plasmid DNA (2 μg) and Superfect (10 μl) for 2 h at 37 °C. Subsequently, the culture media were removed, and the cells were incubated with fresh DMEM containing 10% fetal bovine serum for 24 h to allow for protein expression. The transfected cells were then subjected to serum starvation by incubating in DMEM devoid of fetal bovine serum for 24 h. Transfection efficiency was determined to be 25–30% as assessed by green fluorescent protein cotransfection. Serum-starved cells were pretreated with C2- or C6-ceramide (1 μM) for 1 h and then incubated with PDGF (10 ng/ml) for 5 min (Fig. 6) or 20 h (Fig. 7). For the data shown in Fig. 6, the cell lysates (40 μg of protein) were immunoprecipitated with anti-PKCα antibody, subjected to SDS-PAGE, and then probed with anti-phospho- Akt(Ser473) antibody. For Fig. 7, [3H]thymidine incorporation studies were performed as described above.

Statistical Analyses—The results are expressed as the means ± S.E. of three or more experiments. The data were analyzed by one-way analysis of variance (ANOVA), followed by unpaired t tests, corrected by Bonferroni’s method. In select experiments, non-parametric data were analyzed by Mann-Whitney one-way ANOVA tests, followed by Dunn’s correction method. In those experiments in which the control optical density values were set to 100%, the S.E. for each of these control values was reported using the nontransformed data.

RESULTS

Ceramide Induces Growth Arrest in A7r5 Cells—We have previously shown that ceramide inhibits growth factor receptor-stimulated mitogenesis in rat glomerular mesangial cells (25, 26) and human embryonic kidney cells (4, 5). In the present study, we examined whether ceramide induces growth arrest in rat aortic vascular smooth muscle-derived A7r5 cells. We initially chose a ceramide concentration (10 μM) that was previously shown to maximally induce growth arrest without inducing necrosis or apoptosis. As assessed by [3H]thymidine incorporation studies, cell-permeable ceramide had negligible effects on basal DNA synthesis (Fig. 1). However, pretreatment of A7r5 cells with ceramide led to a significant decrease in PDGF-stimulated DNA synthesis. These results suggest that ceramide inhibits proliferation of A7r5 cells and support our in vitro studies demonstrating that cell-permeable ceramides limit...
over, these data indicate that both C2- and C6-ceramide do not
on Akt activation is not directly dependent upon PI3K. More-
not reveal significant changes in basal or PDGF-stimulated
ments using PI3K assays with intact A7r5 cells revealed that
is mediated through PI3K in A7r5 cells. Our initial experi-
next investigated whether ceramide-induced inhibition of Akt
or IGF-1 (50 ng/ml) stimulation of A7r5 cells leads to maximal
increase in Akt activity (15). Hence, in the present study, we
preincubated without or with 10
M dihydro-C6-ceramide (dihydro-C2-CER) for 2 h, after which both control and treated cells
were stimulated with PDGF (10 ng/ml) for 5 min. The cell lysates were subjected to SDS-PAGE, and the immunoblots were probed with anti-phospho-Akt(Ser473) antibody as described under “Experimental Procedures” for Western blot analyses. The representative protein bands for Akt phosphorylation (pAkt) are shown in the upper panel. The data shown in the lower panel are the means ± S.E. of three to five separate experiments. The arbitrary values shown were obtained by laser densitometric analyses of the protein bands. *, p < 0.05 (repeated measures one-way ANOVA, followed by Bonferroni’s t test).

Ceramide Inhibits PDGF-stimulated Akt Phosphorylation—To determine whether ceramide-induced inhibition of cell growth occurs as a consequence of its regulatory effect on the pro-survival kinase Akt, we examined the effects of ceramide pretreatment on PDGF-stimulated Akt phosphorylation in A7r5 cells. We have previously shown that PDGF (10 ng/ml) or IGF-1 (50 ng/ml) stimulation of A7r5 cells leads to maximal phosphorylation of Akt within 5–10 min, which correlates with increases in Akt activity (15). Hence, in the present study, we used a time point of 5 min to demonstrate Akt phosphorylation in response to PDGF. We now demonstrate that the cell-permeable bioactive C2-ceramide (but not the inactive dihydro-C2-
ceramide) caused significant inhibition of PDGF-induced Akt phosphorylation in A7r5 cells (Fig. 2). These studies suggest that ceramide pretreatment leads to inhibition of growth factor-induced Akt activation in VSMCs.

Ceramide Does Not Inhibit PI3K Activity in A7r5 Cells—We next investigated whether ceramide-induced inhibition of Akt is mediated through PI3K in A7r5 cells. Our initial experiments using PI3K assays with intact A7r5 cells revealed that there was ~3.5-fold increase in the formation of PI3P in response to PDGF stimulation (Fig. 3A). Pretreatment of intact A7r5 cells with either C2- or C6-ceramide, followed by stimulation with PDGF, did not lead to alterations in basal as well as PDGF-stimulated increases in PI3K activity. In addition, dihydro-C6-ceramide did not have any effect on PI3K activity. Similarly, in cell-free experiments, stimulation of A7r5 cells without (control) or with PDGF, followed by treatment of PI3K immunocomplexes with C2-, C6- or dihydro-C6-ceramide, did not reveal significant changes in basal or PDGF-stimulated PI3K activity by ceramide derivatives (Fig. 3B). The data from both intact cell and cell-free experiments indicate that the mechanism by which ceramide mediates its inhibitory effects on Akt activation is not directly dependent upon PI3K. Moreover, these data indicate that both C2- and C6-ceramide do not regulate PI3K at a concentration that we have previously shown to activate PKCζ (4) and to inhibit PKCζ (5).

Ceramide and IL-1β Activate PKCζ in A7r5 Cells—We next investigated an alternative mechanism by which ceramide could inhibit Akt phosphorylation. Based on the previous work of Doornbos et al. (19), we examined whether ceramide may function as the endogenous signal regulating PKCζ-dependent inhibition of Akt. Therefore, we assessed the ability of ceramide to activate PKCζ. Confirming previous studies using other cell types (3, 24, 27), we now demonstrate enhanced PKCζ activity in A7r5 cells in response to ceramide. As shown in Fig. 4, exposure of A7r5 cells to Cζ-ceramide (1 μM, 1 h), but not dihydro-Cζ-ceramide (1 μM, 1 h), resulted in a significant increase in PKCζ activity. We also demonstrate that IL-1β (but not PDGF) could mimic the effects of exogenous ceramide in stimulating PKCζ activity in A7r5 cells. Previously, we (4, 28) and other investigators (29, 30) reported that inflammatory cytokines such as IL-1β induce ceramide formation in multiple cell types. Attesting to the direct action of ceramide upon PKCζ, we have previously shown that ceramide is a cofactor for immunoprecipitated as well as recombinant PKCζ activity (4). Together, these studies show that ceramide activates PKCζ in A7r5 cells.

Ceramide Induces an Association between Akt and PKCζ in A7r5 Cells—As we have shown that ceramide activates PKCζ while inhibiting PDGF-stimulated Akt, we next determined whether ceramide orchestrates a direct interaction between Akt and PKCζ in A7r5 cells. The rationale for utilizing IGF-1 (instead of PDGF) in these experiments was to demonstrate that the effects of growth factors on these interactions are not specific for PDGF. As shown in Fig. 5, we observed a strong selective association of PKCζ (but not PKCs) with Akt after treatment of A7r5 cells with Cζ-ceramide. Dihydro-Cζ-ceramide did not induce such an association of PKCζ or PKCs with Akt. Growth factors such as IGF-1 alone did not exert any effect on the basal association between Akt and PKCζ in A7r5 cells. However, IGF-1 produced a significant inhibition of Cζ-ceramide-induced increases in Akt and PKCζ association. These studies suggest that ceramide may function as an endogenous signal to induce a selective association between PKCζ and Akt in VSMCs.
PKCζ Is a Necessary Component for Ceramide-induced Inhibition of Akt Phosphorylation in A7r5 Cells—The data shown in Fig. 5 demonstrate that ceramide treatment induced the association of PKCζ with Akt. However, a simple interaction between two proteins does not imply direct inhibition of Akt. Therefore, we assessed the critical role of PKCζ in ceramide-induced inhibition of Akt phosphorylation. A7r5 cells were subjected to transient transfection studies utilizing wild-type and dominant-negative mutant PKCζ constructs. In control experiments, there was an increased expression of PKCζ protein in cells transfected with wild-type PKCζ constructs compared with empty vector controls (0.66 ± 0.04 arbitrary units; p < 0.05). In addition, there was no change in the expression of PKCζ protein in cells transfected with wild-type PKCζ constructs compared with empty vector controls (0.10 ± 0.20 versus 0.11 ± 0.04; not significantly different). As shown in Fig. 6, PDGF produced significant increases in Akt phosphorylation in cells overexpressing wild-type PKCζ constructs, consistent with growth factor stimulation of the PI3K pathway. Pretreatment of A7r5 cells (overexpressing wild-type PKCζ) with either C2- or C6-ceramide caused significant attenuations in PDGF-stimulated Akt phosphorylation. In contrast, in cells transfected with the dominant-negative mutant PKCζ constructs, pretreatment with either C2- or DH-C6-ceramide led to significant increases in phosphorylated Akt. Therefore, we assessed the critical role of PKCζ in ceramide-induced inhibition of Akt phosphorylation in A7r5 cells.

Ceramide does not inhibit PI3K activity in A7r5 cells. Intact cell (A) or cell-free (B) PI3K experiments were performed to assess the ability of ceramide to regulate PI3K activity. For A, serum-starved A7r5 cells were pretreated with or without C2-ceramide (C2-Cer; 1 μM), C6-ceramide (C6-Cer; 1 μM), or dihydro-C6-ceramide (DH-C6-Cer; 1 μM) for 1 h and then incubated without or with PDGF (10 ng/ml) for 5 min. The cell lysates were subjected to immunoprecipitation with anti-p85 PI3K antibody, followed by PI3K assays as described under “Experimental Procedures.” For B, serum-starved A7r5 cells were incubated without or with PDGF (10 ng/ml) for 5 min, and the cell lysates were subjected to immunoprecipitation with anti-p85 PI3K antibody. These immunocomplexes were subsequently treated with ceramide analogs (1 μM each) for 10 min at 35 °C. PI3K assays with these ceramide-treated immunocomplexes were then performed as described under “Experimental Procedures.” The data shown are the means ± S.E. of three to six separate experiments. *, p < 0.05 compared with vehicle control (unpaired Mann-Whitney test).

Fig. 3. Ceramide does not inhibit PI3K activity in A7r5 cells. Serum-starved A7r5 cells were exposed to vehicle (Control), dihydro-C6-ceramide (DH-C6-Cer; 1 μM, 1 h), C6-ceramide (C6-Cer; 1 μM, 1 h), IL-1β (20 ng/ml, 5 min), or PDGF (10 ng/ml, 5 min). The cell lysates were subjected to immunoprecipitation with anti-p85 PI3K antibody, followed by PI3K assays as described under “Experimental Procedures.” The data shown are the means ± S.E. of three to four separate experiments. *, p < 0.05 compared with vehicle (repeated measures one-way ANOVA, followed by Bonferroni’s t test).

Fig. 4. Ceramide and IL-1β stimulate PKCζ activity in A7r5 cells. Serum-starved A7r5 cells were exposed to vehicle (Control), dihydro-C6-ceramide (DH-C6-Cer; 1 μM, 1 h), C6-ceramide (C6-Cer; 1 μM, 1 h), IL-1β (20 ng/ml, 5 min), or PDGF (10 ng/ml, 5 min). The cell lysates were subjected to immunoprecipitation with anti-PKCζ antibody, followed by PI3K assays as described under “Experimental Procedures.” The data shown are the means ± S.E. of three to six separate experiments. *, p < 0.05 compared with vehicle (unpaired Mann-Whitney test).

Fig. 5. Ceramide enhances interaction of PKCζ (but not PKCe) with Akt in A7r5 cells. Serum-starved A7r5 cells were pretreated with vehicle (Control), C6-ceramide (C6-Cer; 1 μM, 1 h), or dihydro-C6-ceramide (DH-C6-Cer, DH; 1 μM, 1 h) and then incubated without or with IGF-1 (50 ng/ml) for 5 min. The cell lysates were subjected to immunoprecipitation (IP) with anti-Akt-1 antibody, and the blots were reprobed with anti-PKCζ or anti-PKCe antibody as described under “Experimental Procedures.” To verify equal loading of samples, the blots were reprobed with anti-Akt-1 antibody. The bands shown are representative of a single experiment. The data shown in B are the means ± S.E. of five separate experiments. *, p < 0.001 compared with vehicle control; †, p < 0.005 compared with C6-ceramide treatment alone (unpaired Mann-Whitney test). IB, immunoblot; CO-IP, co-immunoprecipitation.
PKCζ is necessary for ceramide-mediated inhibition of PDGF-stimulated Akt phosphorylation. A7r5 cells were transiently transfected with either wild-type or dominant-negative mutant PKCζ constructs. After transfection using Superfect reagent as described under "Experimental Procedures," the cells were serum-starved for 24 h. Subsequently, these cells were pretreated with C6-ceramide (C6-Cer; 1 mM), C6-ceramide (C6-Cer; 1 mM), or dihydro-C6-ceramide (DH, DH-C6-Cer; 1 mM) for 1 h and then incubated with PDGF (10 ng/ml) for 5 min. The cell lysates were subjected to immunoprecipitation with anti-PKCζ antibody, and the blots were probed with anti-phospho-Akt(Ser473) antibody. To verify equal expression of Akt after transient transfections of A7r5 cells with wild-type or dominant-negative mutant PKCζ, the blots were reprobed with anti-Akt-1 antibody. A shows the representative changes in the intensity of protein bands for Akt phosphorylation (p-Akt) in wild-type versus dominant-negative mutant PKCζ-transfected cells. The data shown in B are the means ± S.E. of three to five separate experiments. *, p < 0.05 compared with vehicle control; #, p < 0.05 compared with PDGF treatment alone (repeated measures one-way ANOVA, followed by Bonferroni’s t test).

FIG. 6. PKCζ is necessary for ceramide-mediated inhibition of PDGF-stimulated Akt phosphorylation. A7r5 cells were transiently transfected with either wild-type (A) or dominant-negative mutant PKCζ (B) constructs. After transfection, the cells were pretreated with vehicle, C6-ceramide (C6-Cer; 1 mM), or dihydro-C6-ceramide (DH-C6-Cer; 1 mM) for 1 h and then incubated with PDGF (10 ng/ml) for an additional 20 h. [3H]Thymidine incorporation into acid-insoluble DNA was determined as described under "Experimental Procedures." The data shown are the means ± S.E. of three to four separate experiments. *, p < 0.05 compared with PDGF treatment alone (repeated measures one-way ANOVA, followed by Bonferroni’s t test).

FIG. 7. PKCζ is necessary for ceramide-mediated inhibition of PDGF-stimulated DNA synthesis. A7r5 cells were transfected with either wild-type (A) or dominant-negative mutant PKCζ (B) constructs. After transfection, the cells were pretreated with vehicle, C6-ceramide (C6-Cer; 1 mM), or dihydro-C6-ceramide (DH-C6-Cer; 1 mM) for 1 h and then incubated without or with PDGF (10 ng/ml) for an additional 20 h. [3H]Thymidine incorporation into acid-insoluble DNA was determined as described under "Experimental Procedures." The data shown are the means ± S.E. of three to four separate experiments. *, p < 0.05 compared with PDGF treatment alone (repeated measures one-way ANOVA, followed by Bonferroni’s t test).

C6- or C6-ceramide had no inhibitory effects on PDGF-stimulated Akt phosphorylation. In all cases, dihydro-C6-ceramide had no effect on Akt phosphorylation. These experiments suggest that PKCζ is a necessary component for ceramide-induced inhibition of Akt activation.

PKCζ Mediates Ceramide-induced Inhibition of Cell Growth—Because we have demonstrated that ceramide-activated PKCζ mediated inhibition of Akt activation, we next examined whether PKCζ is also required for ceramide-induced growth arrest. Therefore, we assessed the effects of ceramide analogs on growth factor-induced DNA synthesis in A7r5 cells overexpressing wild-type or dominant-negative mutant constructs. Transient transfections with wild-type PKCζ led to a significant diminution in PDGF-stimulated DNA synthesis after prior treatment with ceramide (Fig. 7A). These data are in conformity with those shown in Fig. 1. In cells overexpressing dominant-negative mutant PKCζ constructs, pretreatment with C6-ceramide had no significant inhibitory effects on PDGF-stimulated DNA synthesis (Fig. 7B). Dihydro-C6-ceramide was without any effect on PDGF-stimulated DNA synthesis after overexpression of either construct. Thus, these observations demonstrate that ceramide-activated PKCζ leads to marked inhibition of growth factor-induced Akt activation as well as cell growth, independent of PI3K.

DISCUSSION

In addition to its well-established role as a cell survival kinase, Akt has been implicated in cell proliferation in different cell types, including VSMCs (13–16, 31–34). Activation of Akt at the membrane is a highly regulated event involving several lipid-regulated pleckstrin homology domain-containing kinases as well as PI3K-generated phosphoinositide-3-phosphate derivatives (13, 35–39). These events lead to conformational changes in Akt-1, with subsequent phosphorylation at Thr308 and Ser473. Fully activated, phosphorylated Akt is able to translocate to the nucleus, where it regulates the protein synthesis of numerous pro-mitogenic and pro-survival transcription factors (40–44).

Recent studies have suggested that the endogenous sphingolipid-derided second messenger ceramide induces growth arrest, in part, via dephosphorylation of Akt (9, 10, 45). However, the mechanism leading to inhibition of Akt has not been conclusively demonstrated. As ceramide does not directly inhibit Akt (7), multiple indirect mechanisms for ceramide regulation of Akt have been proposed. An obvious potential mediator for ceramide-induced inhibition of Akt is PI3K. Conflicting results have suggested PI3K-dependent (6) as well as PI3K-independent (17, 18) regulation of Akt by ceramide. There are also several reports demonstrating activation of Akt, independent of PI3K (46, 47). Our studies argue for a PI3K-independent mechanism for ceramide-induced Akt inhibition in VSMCs.
PKCζ has been implicated as a putative mediator of PI3K-independent Akt inactivation (19). It has been suggested that PKCζ is a negative regulator of PDGF-induced increases in Akt activity (19). The ability of PKCζ to negatively regulate Akt may imply that an interaction between PKCζ and Akt abrogates the phosphorylation of Akt, yet the precise inhibitory/regulatory sites for PKCζ-dependent phosphorylation of Akt currently remain undefined. In addition, the endogenous regulator of PKCζ-dependent inhibition of Akt has not been determined. These studies suggest that ceramide may function as one such endogenous mediator, regulating PKCζ-dependent inhibition of Akt phosphorylation in VSMCs. Our studies further support the biological significance of PKCζ as one of the potential targets for ceramide.

The role of ceramide in binding to and activating PKCζ is still somewhat controversial. It has been hypothesized that ceramide directly interacts with the single cysteine-rich domain of PKCζ (26, 48). Our previous studies using both immunoprecipitated PKCζ and human recombinant PKCζ clearly demonstrate that ceramide (but not dihydroceramide) directly induces PKCζ bioactivity (4). Supporting our findings, ceramide has been shown to bind to PKCζ as determined by kinetic analyses and in vitro phosphorylation studies (24, 27). In contrast, a radiiodinated photoaffinity-labeled ceramide analog was unable to directly interact with immunoprecipitated PKCζ (49). These apparent contradictions in the literature may be due to structural differences in the ceramide analogs utilized. Even if ceramide does not directly interact with PKCζ, our studies document that ceramide treatment leads to a selective interaction between Akt and PKCζ and that activated PKCζ is necessary for ceramide-induced inhibition of Akt.

In addition to the controversy surrounding ceramide-stimulated PKCζ, there is disagreement regarding the signaling function of PKCζ. There are several reports in the literature that suggest that PKCζ mediates mitogenic signaling pathways (50–53). These studies discuss PKCζ as a downstream signaling target for PI3K and phosphoinositide 3,4,5-trisphosphate-dependent kinase-1. Other reports suggest that PKCζ may exert growth-arresting effects by directly inhibiting Akt (19, 20). Our present studies, using dominant-negative mutant PKCζ constructs, firmly suggest that ceramide activation of PKCζ in VSMCs leads to growth arrest. It is also possible that PKCζ may serve as a bifunctional modulator, as suggested by Muller et al. (24). Thus, it is possible that ceramide and phosphoinositide-3-phosphate derivatives may differentially activate and couple PKCζ to both pro- and anti-mitogenic effectors. This is a similar scenario to previously published data demonstrating that ceramide preferentially couples activated PKCζ to upstream elements in the anti-mitogenic stress-activated protein kinase cascade, and not the pro-mitogenic extracellular signal-regulated kinase cascade (4).

PKCζ may not be the only target for ceramide that inhibits Akt. Phosphoinositide 3,4,5-trisphosphate-dependent kinase-1, an upstream kinase for both Akt (41, 54) and PKCζ (51, 52, 55), could also be regulated by ceramide. However, our studies demonstrating that ceramide inhibition of Akt is PI3K-indepen-dent suggest that phosphoinositide 3,4,5-trisphosphate-dependent kinase-1 may not be a target for ceramide regulation. In fact, a recent report demonstrates that ceramide does not affect phosphoinositide 3,4,5-trisphosphate-dependent kinase-1 activity (10).

In addition to activation of PKCζ, ceramide has been suggested to activate protein phosphatases that may dephosphorylate Akt, thereby preventing translocation into the nucleus (10, 17, 18). Even though we did not directly assess the putative role of ceramide-activated protein phosphatases in mediating PKCζ-dependent inhibition of Akt, studies demonstrate that ceramide activates PKCζ under conditions in which ceramide activates protein phosphatases (10, 56, 57). Yet, the literature regarding the role of ceramide-activated protein phosphatases in regulating Akt inhibition is somewhat controversial. Ceramide has been shown to dephosphorylate Akt-1 at Ser473 and Thr308 suggesting a role for a ceramide-activated protein phosphatase (9, 10). However, studies by Zhou et al. (7) and Sum-mers et al. (8) suggest that ceramide-induced inactivation of Akt is independent of protein phosphatases. These discrepancies in the literature may be a consequence of nonspecific or toxic actions of the pharmacological inhibitors of protein phosphatase-1 and -2A utilized in these studies. Using a molecular approach, it has been suggested that ceramide inhibition of Akt occurs via a protein phosphatase-independent mechanism (19). Specifically, PKCζ was able to inhibit the activity of a constitutively active Akt-1 mutant that is not regulated by phosphorylation/dephosphorylation at Ser473 and Thr308.

The mechanism(s) by which growth factors inhibit ceramide-induced increases in the interactions of PKCζ with Akt remains to be verified. Establishing ceramide as a mediator of PKCζ/Akt interactions offers several possible explanations for this observation. Growth factor-generated diglycerides may compete with ceramide at the putative ceramide-binding site on PKCζ (58) or activate other PKC isoforms linked to mitogenesis. Alternatively, growth factor treatment induces the activation of ceramidase, which forms pro-mitogenic sphingosine species (28, 59). The ability of growth factors to inhibit ceramide-activated PKCζ/Akt interactions probably does not involve activation of PKCζ because we (this study) and others (19, 60) have shown that growth factors induce a marginal increase in PKCζ activity. Regardless of the mechanism, growth factor treatment reduces the actions of inflammatory cytokines or ceramides in inducing PKCζ/Akt interactions.

We have shown that ceramide-induced inhi-bitions of Akt activation and cell growth are mediated by PKCζ activation, independent of PI3K. The role of ceramide in selectively inducing PKCζ/Akt complex formation may illustrate one mechanism by which cytokine receptor-induced ceramide formation may limit cell proliferation in a pro-inflammatory environment. Inhibition of Akt by cell-permeable ceramide analogs may have direct applicability to the control of dysregulated VSMC proliferation in vivo.

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