Calphostin-C Induction of Vascular Smooth Muscle Cell Apoptosis Proceeds through Phospholipase D and Microtubule Inhibition*

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Calphostin-C, a protein kinase C inhibitor, induces apoptosis of cultured vascular smooth muscle cells. However, the mechanisms are not completely defined. Because apoptosis of vascular smooth muscle cells is critical in several proliferating vascular diseases such as atherosclerosis and restenosis after angioplasty, we decided to investigate the mechanisms underlying the calphostin-C-induced apoptotic pathway. We show here that apoptosis is inhibited by the addition of exogenous phosphatidic acid, a metabolite of phospholipase D (PLD), and that calphostin-C inhibits completely the activities of both isomers of PLD, PLD1 and PLD2. Overexpression of either PLD1 or PLD2 prevented the vascular smooth muscle cell apoptosis induced by serum withdrawal but not the calphostin-C-elicited apoptosis. These data suggest that PLDs have anti-apoptotic effects and that complete inhibition of PLD activity by calphostin-C induces smooth muscle cell apoptosis. We also report that calphostin-C induced microtubule disruption and that the addition of exogenous phosphatidic acid inhibits calphostin-C effects on microtubules, suggesting a role for PLD in stabilizing the microtubule network. Overexpressing PLD2 in Chinese hamster ovary cells phenocopies this result, providing strong support for the hypothesis. Finally, taxol, a microtubule stabilizer, not only inhibited the calphostin-C-induced microtubule disruption but also inhibited apoptosis. We therefore conclude that calphostin-C induces apoptosis of cultured vascular smooth muscle cells through inhibiting PLD activity and subsequent microtubule polymerization.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Calphostin-C and GF 109203X were obtained from Calbiochem. Monoclonal antibodies for α-tubulin, phosphatidic acid, and propidium iodide (PI) were purchased from Sigma. RPMI 1640 medium and fetal bovine serum were purchased from Invitrogen. Cell Culture and Treatment—Primary culture of VSMCs was carried out using the explant method as described previously (20). The blood vessel tissues were derived from thoracic aorta of male Sprague-Dawley rats (250–300 g). The medial aortic tissue was cut into small pieces (1 mm³), which were then placed on the plastic surface of culture dishes. The tissue explants were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The culture was carried out at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were usually observed to migrate from tissues between days 4 and 7 after explanting. The islets of outgrowing cells were then treated with 0.25% trypsin and 0.25 mM EDTA solution (Invitrogen) followed by subculture. The cells from passage 6–8 were used for the experiments described. Calphostin-C was dissolved in water and added to culture medium directly to achieve a final concentration of 0.2 μM for 24 h. To test the effects of PA on calphostin-C-induced apoptosis, PA micelles (1 μg/ml) were added to the culture medium.
culture medium 30 min before calphostin-C. In serum starvation experiments, cells were cultured in medium containing 0.5% serum for 2 days before any treatment. CHO cells were cultured using Ham’s F-12 medium supplemented with 10% (v/v) fetal calf serum essentially like VSMCs. These cells harbored either hemagglutinin-PLD2 or hemagglutinin-PLD2-K758R under control of a tetracycline-inducible T-Rex (Invitrogen). Expression of PLD2 or PLD2-K758R was induced by adding 1 μg/ml doxycycline (Dox) for 20 h.

Apopotis and DNA Analysis Using Laser-scanning Cytometry (LSC)—VSMCs were cultured on acid-washed coverslips. After various treatments, cells were fixed with 80% ethanol for 20 min and cold 100% ethanol for another 20 min. After rinsing with PBS (50 mM NaH2PO4, 300 mM NaCl, pH 7.4), cells were incubated with a solution containing 5 mg/ml PI and 0.1% Triton X-100 for 20 min. Coverslips were mounted with Prolong (Invitrogen). DNA analysis and apoptosis of VSMCs in response to calphostin-C were performed with LSC (CompuCyte, Cambridge, MA) as previously described (21, 22). Data were acquired and analyzed with WinCyte acquisition software (version 3.4, CompuCyte). Apoptosis of cultured VSMCs was indicated by the appearance of a sub-G0/G1 apoptotic peak. The apoptotic cells in the sub-G0/G1 peak were confirmed by using relocation of LSC to observe cells directly through the CCD camera.

DNA Fragmentation Assay—After treatment with or without 0.2 μM or 1 μM calphostin-C for 24 h, cells were collected and solubilized by vigorous vortexing in TE95 buffer (0.5 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 9), 1% (w/v) SDS) containing proteinase K (1 mg/ml). This was followed by a 3-h incubation at 50°C and DNA extraction using phenol-chloroform. After incubation with RNase A (1 mg/ml) for 1 h at 20–22°C, DNA samples were analyzed using conventional electrophoresis in 1.5% (w/v) agarose gels. The gels were visualized using a gel documentation system (Bio-Rad).

GFP-PLD Expression Vector Construction and the Establishment of Stably Transfected Cells—The construction of pCGN-PLDib (GenBank™ accession U38554), pCGN-PLDib-K898R (inactive allele), pCGN-PLD2 (GenBank™ accession U87557), and pCGN-PLD2-K758R (inactive allele) was performed as described previously (23, 24). All of the full-length CDNa were subcloned into pEGFP vectors. To avoid changing the reading frames, PLDib and PLD2 were cloned into pEGFP-N2 and pEGFP-C1 vectors, respectively (Clontech Laboratories). PLDib and PLD1-K898R were excised from pCGN-PLDib and pCGN-PLD1-K898R with HapI and SmaI. PLD2 and PLD2-K758R were excised from pCGN-PLD2 and pCGN-PLD2-K758R with XbaI and SmaI. Both pEGFP-C2 and pEGFP-C3 were digested using SmaI. Excised cDNA fragments were purified using a gel extraction kit (Qiagen). The sizes of the excised fragments were confirmed by gel electrophoresis. After construction, restriction mapping confirmed the presence and orientation of the PLDib and PLD2 cDNAs in the vector.

Cells were transiently transfected with each DNA preparation using LipofectAMINE 2000 according to the instructions provided by the manufacturer (Invitrogen). Successful transfection was confirmed by fluorescence microscopy. To make stably transfected cells, transfected cells were selected by adding 400 ng/ml G418 into the culture medium. After forming single colonies in the presence of G418, all of the cells were pooled to make a stable cell mixture and these cells were maintained in 100 ng/ml G418, which was withdrawn 1 week before setting up the experiments. The expression of PLDib in stably transfected cells was confirmed using both immunoprecipitation/Western blot and a PLD activity assay.

Immunoprecipitation and Western Blot Analysis of GFP and PLD-GFP Expression—Stably transfected cells after 24-h serum starvation were lysed for protein extraction. An equal amount of protein (100 μg) from each sample was added to 1 ml of PBS followed by an overnight incubation with 50 μl of the anti-GFP (G2604) and protein G-Sepharose beads at 4°C. Immunoprecipitated proteins were analyzed using standard SDS-PAGE. Proteins were then transferred (30 V, overnight, 4°C) from the gels to a nitrocellulose membrane. Membranes were blocked with 10% (w/v) bovine serum albumin for 1 h at room temperature followed by incubation with anti-GFP mouse monoclonal antibody to detect both GFP and PLD-GFPs. A horseradish peroxidase-coupled goat anti-mouse IgG was used as the secondary antibody. Visualization of signals was achieved by using chemiluminescence (ECL reagent, Amersham Biosciences).

PLD Activity Assay—Culture medium containing 0.5% serum for 2 days was then labeled with 1 μCi/ml [9,10(-3H)]-myristic acid (Amersham Biosciences, catalog number TM-025). The labeled medium was then washed with PBS, pre-equilibrated at 37°C for cell starvation medium for 60 min. Butanol (0.5%) was included during the last 10 min of incubation. After termination of the reaction, cells were collected and fractionated and separated with 0.6 ml of methanol/chloroform/0.1 N HCl (v/v/v, 1:1:1). A fraction of the extracted lipids was measured for total radioactivity using a liquid scintillation counter (Beckman). Samples were subsequently separated on a silica gel TLC sheet. After autoradiography, the spots corresponding to the reference lipid (phosphatidylbutanol) were scraped to scintillation vials for the measurement of radioactivity. The activities of PLDs were presented as a ratio of radioactivity for each spot to the total radioactivity.

Immunocytochemical Studies—Cells cultured on coverslips were treated with or without calphostin-C for 48 h. These cells were then rinsed with PBS and fixed with 80% ethanol for 20 min. Cells were subsequently permeabilized with 0.25% Triton X-100 in PBS. After incubation with bovine serum albumin (0.5%) containing PBS for 20 min, cells on coverslips were incubated with a primary antibody to target the protein of interest, washed with PBS, and incubated with Alexa Fluor-488 labeled secondary antibodies. Cell nuclei were counterstained with a PI solution (5 μg/ml) that contained RNase (200 ng/ml). Cellular fluorescent signals were detected using either regular fluorescence microscopy or LSC. CHO cells after induction of PLD2 and PLD2-K758R with Dox (1 μg/ml) for 20 h were fixed with cold methanol following by staining for α-tubulin and overexpressed PLD2.

Data Analysis—Data are expressed as the mean ± S.E. The number of replicate (n) represents the number of cells used in the studies. Differences between means were evaluated by the Student’s t test (paired or independent) when two groups were compared and by analysis of variance (ANOVA) followed by Bonferroni’s correction when three or more groups were compared. p < 0.05 or p < 0.01 was considered significant. All statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS, version 10, Chicago, IL).

RESULTS

Smooth Muscle Cell Apoptosis Induced by Calphostin-C—We had observed that there was significant cell death with a slight rounding up of cultured rat VSMCs in response to treatment with calphostin-C (0.2 μM) but not GF109203X (3 μM) (data not shown). It has been reported that calphostin-C induces apoptosis in several cell systems including VSMCs in culture. Therefore, we wished to confirm this calphostin-C-induced apoptosis in cultured rat aortic VSMCs. To do so, we extracted DNA from cells with or without treatment with calphostin-C (0.2 or 1 μM) for 24 h. The DNA samples were analyzed using agarose gels as described under “Experimental Procedures.” Our results showed that calphostin-C at 0.2 μM induced significant DNA fragmentation, and the fragmentation was even more prominent at a concentration of 1 μM (Fig. 1, panel A). Typical apoptotic nuclei in response to calphostin-C treatment were observed directly with fluorescent microscopy after PI staining (Fig. 1, panels B and C). We further analyzed VSMC apoptosis induced by calphostin-C with LSC, a microscope-based cytometer. As shown in Fig. 1, panels D and E, we observed the typical sub-G0/G1 apoptotic peak in the DNA histograms. Cells in the apoptotic peak were then located using LSC relocation to confirm their apoptotic morphologies as shown in the insert of Fig. 1, panel E. Accumulated data showed that apoptotic cell number (percentage of total cells) significantly increased in response to treatment with calphostin-C (0.2 μM). The apoptosis in VSMCs induced by calphostin-C appeared independent of its PKC inhibition, because inhibition of PKC by GF109203X did not induce an apoptotic response (data not shown). Recently, calphostin-C has been reported to directly inhibit the activities of PLD independent of its inactivation of PKC (8). Given that PLD has anti-apoptotic effects in some cell systems, we hypothesized that inhibition of PLD could contribute to the apoptotic action of calphostin-C. Therefore, we explored the following two sets of experiments to test this possibility.

Inhibition of PLD Activities by Calphostin-C—To test the involvement of PLDs in calphostin-C-induced apoptosis, we first examined whether calphostin-C inhibits PLD activities in cultured rat aortic VSMCs. To do so, we labeled the lipid pool with [9,10(-3H)]-myristic acid and used butanol for transphosphatidylation to measure PLD activities of cells cultured with or without the presence of calphostin-C at 0.2 μM for 24 h. As

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expected, calphostin-C treatment completely inhibited the activity of PLDs in cells cultured with 10% serum (Fig. 2). Therefore, this observation has confirmed that calphostin-C is a potent PLD inhibitor in VSMCs. Also, it suggests that PLD inhibition may be the mechanism through which calphostin-C induces apoptosis in VSMCs. To further test this hypothesis, we examined the effect of exogenous phosphatidic acid on calphostin-C-induced apoptosis.

PA Inhibits Calphostin-C-induced Apoptosis—Cultured VSMCs were incubated with phosphatidic acid micelles (1 μg/ml) 30 min before treatment with calphostin-C (0.2 μM) for 24 h. As anticipated, the results showed that treatment of cells with PA prevented the appearance of the sub-G0/G1 apoptotic peak induced by calphostin-C as shown in the DNA histograms acquired using LSC (Fig. 3). This inhibition of apoptosis by PA was confirmed using the DNA fragmentation assay (data not shown). These data, along with the results that show PLD inhibition by calphostin-C, strongly suggested that calphostin-C induced apoptosis of cultured VSMCs through its inhibition of PLDs. However, we wished to establish further that PLD activation in cultured VSMCs indeed has an anti-apoptotic effect.

Overexpression of Either PLD1b or PLD2 in Cultured VSMCs Inhibits Apoptosis Induced by Serum Starvation—In our pre-

**Fig. 1. Characterization VSMC apoptosis induced by calphostin-C.** Rat VSMCs were cultured with or without calphostin-C for 24 h as described under “Experimental Procedures.” Apoptosis was characterized by a DNA fragmentation assay (A), direct observation using fluorescent microscopy (B and C), and LSC (D and E). Panel A shows a DNA-agarose gel for the DNA fragmentation assay in which M indicates the DNA marker and C is the control. Cells were treated with calphostin-C at concentrations of 0.2 and 1 μM (lanes 3 and 4) for 24 h. Panels B (control cells) and C (treated with calphostin-C, 0.2 μM for 24 h) are photomicrographs showing nuclear staining with PI. Cells are stained with a PI solution containing RNase A (200 ng/ml). Panels D and E are DNA histograms derived from LSC. In panel D, the y axis (Count) shows the number of scanned cells and the x axis (PI Integral) shows the total DNA in each cell. Cells in region 1 are considered to be apoptotic cells. In panel E, the peak in region I is the sub-G0/G1 apoptotic peak. The cells in this peak are relocated to observe their morphologies to confirm that they are apoptotic cells (inset). Panel F is a bar figure for accumulated data in which the y axis is the percentage of apoptotic cells in each condition derived from LSC. The x axis indicates the different groups. The asterisk indicates a significant difference (p < 0.01).

**Fig. 2. Inhibition of PLD activity by calphostin-C.** The PLD activities in VSMCs cultured in medium containing 10% serum are represented by the formation of phosphatidylbutanol as described under “Experimental Procedures.” The y axis indicates the total PLD activity as a percentage of that in control cells without any treatment. Results are presented as mean ± S.E. (n = 4). The PLD activity of control cells is set as 100. The x axis indicates different groups with various treatments.
liminary studies, we observed that serum withdrawal induced apoptosis of cultured rat aortic VSMCs as described by others (25). It has been reported that cultured cells have low PLD activities in the absence of serum (26). The addition of exogenous PA prevented the apoptosis of cultured VSMCs, resulting from serum starvation for 3 days (Fig. 4, panel A). Therefore, we hypothesized that overexpression of either PLD1b or PLD2 would prevent apoptosis resulting from serum withdrawal. To test this possibility, we established stably transfected cells harboring PLD1b, PLD2, or their inactive forms (see “Experimental Procedures”). The expression of PLD-GFP fusion proteins was first confirmed by observing the bright fluorescence in transiently transfected cells (data not shown). Cells were then selected with G418 (400 ng/ml) for 2 weeks. The expression of PLD-GFP fusion proteins in stably transfected cells was further confirmed using immunoprecipitation/Western blot to detect the presence of GFP as shown in Fig. 4, panel B. As anticipated, our data showed that control cells harboring only GFP undergo significant apoptosis after 3 days of culture in serum-free medium as analyzed using LSC. Cells in each condition derived from LSC. The x axis indicates the different groups. The asterisk indicates a significant difference (p < 0.01).

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Exogenous PA inhibits calphostin-C-induced apoptosis of VSMCs. Cells were cultured on coverslips without or with the presence of calphostin-C (panels A and B) for 24 h followed by analysis using LSC. Cells in panel C were pretreated with PA (1 μg/ml) before the addition of calphostin-C (0.2 μM). The y axis (Count) shows the number of scanned cells, and the x axis (PI Integral) shows the total DNA in each cell. Cells in region 1 are considered to be apoptotic cells. Panel D is a bar figure for accumulated data in which the y axis is the percentage of apoptotic cells in each condition derived from LSC. The x axis indicates the different groups. The asterisk indicates a significant difference (p < 0.01).

Calphostin-C Induction of Microtubule Disruption and Effects of PA and Taxol—To address whether calphostin-C modulates the microtubule network, we first examined microtubules via immunostaining for α-tubulin. As expected, calphostin-C treatment induced significant disruption of microtubules (Fig. 5, panel B). Importantly, the addition of exogenous PA prevented the disruption of microtubules and the morphologic changes (Fig. 5, panel C). As expected, a microtubule stabilizer, taxol (0.01 μM) (27–29), suppressed the effect of calphostin-C on microtubules (Fig. 5, panel D). These data strongly suggest that the activity of PLD plays important roles in microtubule polymerization. To further evaluate this possibility, we overexpressed PLD2 and its inactive form in CHO cells using a Dox-inducible system (data not shown) and found that overexpression of PLD2 but not PLD2K758R, an inactive form of PLD2, promoted the formation of elongated and parallel-aligned microtubules in CHO cells (Fig. 5, panel E). This finding therefore provides direct evidence to support the hypothesis that PLD activity contributes to the regulation of the microtubule network. As predicted, stabilization of the microtubules using taxol (0.01 μM) inhibited the apoptosis of cultured VSMCs in response to treatment with calphostin-C (Fig. 6).
Fig. 4. Overexpression of PLD1b and PLD2 inhibits apoptosis induced by serum withdrawal. Serum starvation-induced apoptosis was prevented by the presence of PA (panel A). Cells on coverslips were cultured in serum-free medium without (control) and with (+PA) the presence...
DISCUSSION

In this report, we have investigated the mechanisms underlying VSMC apoptosis in response to calphostin-C treatment. Our novel finding is that PLD in VSMCs has an anti-apoptotic effect and that calphostin-C completely inhibits PLD activity in VSMCs, which results in apoptosis. Importantly, we have for the first time provided evidence that complete inhibition of PLD activity induces microtubule disruption, contributing to the apoptotic response of cultured VSMCs triggered by calphostin-C.

VSMC apoptosis has been observed in various cardiovascular diseases including hypertension, restenosis, and atherosclerosis (30–35). Therefore, the investigation of VSMC apoptosis may contribute to our understanding of these vascular diseases. It is important to know what induces apoptosis, but it is equally important to explore how apoptosis is induced. Calphostin-C was reported to induce apoptosis in VSMCs via undefined mechanisms. To explore the underlying mechanisms, we first confirmed the VSMC apoptotic response to calphostin-C. We verified this using three different approaches including DNA fragmentation, direct observation of apoptotic morphology, and LSC. Although calphostin-C is an inhibitor of PLD (1), cells were treated without (A) or with (B) calphostin-C (0.2 μM) for 24 h followed by fixation and immunostaining for fluorescence microscopy. Panels C and D were cells pretreated with PA (1 μg/ml) and taxol (0.01 μM) before the addition of calphostin-C. Panel E shows that Dox-induced overexpression of PLD2 (upper panels), but not PLD2K758R (lower panels), in CHO cells results in elongation and formation of parallel-aligned microtubules. WT, wild type.

FIG. 5. Calphostin-C induction of microtubule disruption in VSMCs and effects of overexpressed PLD2 on microtubules in CHO cells. Cultured VSMCs (panels A–D) and CHO cells (panel E) were stained for α-tubulin as described under “Experimental Procedures.” VSMCs were treated without (A) or with (B) calphostin-C (0.2 μM) for 24 h followed by fixation and immunostaining for fluorescence microscopy. Panels C and D were cells pretreated with PA (1 μg/ml) and taxol (0.01 μM) before the addition of calphostin-C. Panel E shows that Dox-induced overexpression of PLD2 (upper panels), but not PLD2K758R (lower panels), in CHO cells results in elongation and formation of parallel-aligned microtubules. WT, wild type.
PKC, its induction of apoptosis is not through its inhibition of PKC because we observed that inhibition of PKC by GF109203X did not induce apoptosis. Therefore, we pursued exploration of PKC-independent pathways to define the calphostin-C-mediated apoptotic effect.

From our results, we conclude that PLD inhibition mediates calphostin-C induction of apoptosis in cultured VSMCs. This conclusion is based on the following evidence. First, calphostin-C completely inhibits PLD activity in cultured VSMCs, in keeping with data from other cell systems (36). Second, the addition of exogenous PA, the enzymatic product of PLD, reverses the calphostin-C apoptotic effects. Third, overexpression of either PLD1b or PLD2 in VSMCs inhibits the VSMC apoptosis induced by serum withdrawal. This PLD anti-apoptotic effect has been proposed for other cell systems as well (9–12).

Our data show that exogenous PA inhibits calphostin-C-induced apoptosis and that either PLD1b or PLD2 overexpression inhibits apoptosis. It appears that PA from either PLD1b or PLD2 has anti-apoptotic effect in cells. It is well known that PA is a key signaling molecule in cells. Therefore, it is not surprising that deprivation of PA will induce cell death. Because PA can trigger many signaling pathways in cells, it has been a great challenge to identify how PA produced by PLDs allows cells to survive. Our finding that PLD inhibition stimulates microtubule disruption points out a new avenue to identify the roles of PLDs in cell biology. In support of this proposal, we demonstrated directly by overexpressing PLD2 in CHO cells that PLD activity modulates microtubule structures. Therefore, our findings imply that PLD activity is required for the formation of a functional cytoskeleton. We speculate that endogenous PA, produced through PLD activation, directly or indirectly participates in maintaining the integrity of the microtubule network. One possibility is that PA (or its metabolites) contributes to the assembly of microtubules. These observations raise many questions regarding PLDs and the microtubule network.

PLD1 directly binds to actin, and its activation induces the formation of stress fibers (16–18). It is now clear that actins may cross-talk with microtubules at focal adhesion structures. However, we do not know whether PLD1 bound to actins or PA produced from this enzyme modulates the microtubule structure and/or function. More studies are required to clarify whether such cross-talk occurs in mammalian cells. A recent study using plant cells reported that a 90-kDa PLD binds directly to microtubules and that this enzyme can be regulated in response to microtubule assembly (19). Presumably, PA produced from this enzyme is acting on the microtubules.

In a separate study, we identified that microtubule disruption induced VSMC apoptosis and investigated the underlying mechanisms. In the current study, taxol, a stabilizer of microtubules (27–29), inhibited the apoptosis induced by calphostin-C, providing further evidence to support the role of microtubules in VSMC apoptosis. In summary, we have demonstrated that PLD inhibition induced by calphostin-C results in microtubule disruption of VSMCs and subsequent apoptosis.

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