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Role of Protein Kinase C δ in an Early Stage of Coxsackievirus-B3-Induced Apoptosis in HeLa Cells

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Abstract CVB3 is a virulent human pathogen that induces myocarditis and ultimately dilated cardiomyopathy. Although several apoptotic factors are involved in the cell death induced by CVB3, the upstream signal transduction factors of CVB3-induced apoptosis are still unclear. We explored and characterized the role of PKC δ in CVB3-infected cells. PKC δ was cleaved after CVB3 infection and was activated at 6 h postinfection. PKC δ was also translocated into the nucleus via mitochondria after CVB3 infection, and overexpression of wild-type PKC δ reduced the apoptotic cell death caused by CVB3. These results indicate that PKC δ has an anti-apoptotic role in CVB3 infection.

Key words: CVB3, PKC δ, apoptosis

CVB3 is one of the picornaviruses with a single-stranded positive-sense RNA genome of about 7,400 nucleotides. Its genome encodes four capsid proteins and seven nonstructural proteins [24]. CVB3 induces acute and chronic viral myocarditis in children and young people [13], with dilated cardiomyopathy at its end-stage, causing about 5 to 50% of all cases [19]. However, the pathophysiological mechanism of viral myocarditis is still a matter of debate. The apoptosis directly induced by CVB3 and the indirect autoimmune responses induced by cytokines in the infected heart remain attractive candidate mechanisms, but are as yet unconfirmed hypotheses [10, 25]. Viral protease 2A cleaves eIF4-G and inhibits the synthesis of host proteins, thus inducing apoptosis [7, 16]. Moreover, HeLa cells infected with CVB3 show cleavage of caspase, the release of cytochrome *c*, and the degradation of PARP. Overexpression of Bcl-2 and Bcl-xL blocks this cytochrome *c* release, inhibits caspase cleavage, and finally reduces the cell death caused by viral infection [5]. However, it is still unclear which upstream signal transduction factors regulate apoptotic cell death after CVB3 infection.

It is generally accepted that PKC, which comprises at least 10 isoforms, exerts both inhibitory and stimulatory effects on apoptosis. Although there are some exceptions, much evidence indicates that PKC α, β, and ε and atypical isoforms are anti-apoptotic, whereas the δ and θ isoforms are usually involved in the promotion of apoptosis [8]. In particular, PKC δ and ε play critical balancing roles in protecting and/or damaging the heart [1, 11, 21]. Furthermore, these two isoforms are subjected to restrictive proteolysis during apoptosis [15]. Therefore, it is reasonable to expect that PKC δ and ε are involved in CVB3-induced apoptosis. In this study, we characterized the regulation of PKC δ in HeLa cells infected with CVB3.

Materials and Methods

Cells, Viruses, and Recombinant Plasmids

The cervical HeLa-UVM cancer cell line [3] (here simply termed HeLa) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum as previously reported [18]. CVB3, an H3 Woodrupt strain (donated by Professor E. Jeon, Samsung Medical Center, Seoul, Korea), was grown and titered using HeLa cells. Viral stocks were prepared by infecting 90% confluent cultures of HeLa cells at a multiplicity-of-infectivity (moi) of 10. After infection for 16 h at 37°C, the suspension was freeze-thawed three times. The cell lysates were centrifuged and the supernatants were harvested and stored at −75°C. The amount of virus was measured by

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plaque assay. The titer of CVB3 H3 was 1×10^9 plaque-forming unit (pfu)/ml.

Two recombinant plasmids encoding CVB3 2A and 3C were constructed from infectious cDNA of CVB3 (H3 strain). All 5' sense primers included the Kozak sequence (bold) for translation efficiency and a start codon (**CACC**ATG), and 3' antisense primers included a stop codon (TAA). PCR products were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, MD, U.S.A.).

Western Blot Analysis

Western blotting analysis was done as previously reported [2, 14]. Cell monolayers were washed with Tris-buffered saline (TBS) and scraped into ice-cold TBS with a rubber policeman. Cells were lysed in SDS-PAGE sample buffer and subjected to SDS-PAGE on 10% acrylamide gels. Samples were transferred to nitrocellulose by semi-dry blotting and membranes were blocked in 5% skim milk. The blots were then analyzed by incubation with appropriate antibodies directed against PKC δ (Santa Cruz, CA, U.S.A.), PKC ε (Upstate, VA, U.S.A.), VP1 (Novo-castra, UK), caspase-3 (Santa Cruz, CA, U.S.A.), and tubulin (Abchem, U.K.), followed by corresponding secondary antibody and developed using ECL reagents (Amersham Pharmacia, MA, U.S.A.).

Collection of Cytosolic and Nuclear Fractions of CVB3-Infected Cells

CVB3-infected HeLa cells were lysed with M-PER buffer (Pierce, IL, U.S.A.), and lysate was centrifuged. The supernatant was collected as the cytosolic fraction and the pellet was lysed with 1% Triton X-100 in PBS containing protease inhibitors. Each fraction was subjected to Western blotting analysis for the full-length (FL) and catalytic fragment (CF) forms of PKC δ.

Immunoprecipitation

After CVB3 infection, cells were harvested at the indicated times in lysis buffer (1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, and 1 mM leupeptin in kinase buffer). Proteins were immunoprecipitated with anti-PKC δ and Sepharose A bead (Amersham Pharmacia, MA, U.S.A.), and then washed three times with kinase buffer [20 mM Tris (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, and 1 mM dithiothreitol]. The pellet was incubated with 2 μCi γ-^{32}P ATP, 50 μg/ml phosphatidyl-serine, and 5 μg of Histone H1 for 20 min at 30°C. SDS sample buffer was added, and the samples were resolved on SDS-PAGE.

Fluorescence-Activated Cell Sorting (FACS) Analysis

FACS analysis was done as previously reported [12]. Cell death was analyzed by staining with annexin V-FITC. At 24 h after transfection, cells were infected for 4–10 h. Fixed cells (1×10^6) were harvested by centrifugation and washed twice with PBS. Washed cells were suspended in 200 μl of 1×PBS containing 1mM EDTA. These cells were stained with 100 μg/ml annexin V-FITC and analyzed on a FACS Vantage flow cytometer (Beckton Dickinson, San Jose, CA, U.S.A.).

Immunofluorescence

In the presence or absence of inhibitor, 60% confluent monolayers of HeLa cells were grown on glass coverslips. The cells were then infected at a moi of 20 CVB3 at 37°C for various times. The cells on glass coverslips were fixed with 4% paraformaldehyde for 15 min, quenched with 50 mM NH_4Cl for 10 min, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were washed, blocked with PBA (containing 2% BSA), and incubated with primary antibody (1:50–1:100 dilution) for 40 min. Subsequently, the coverslips were washed three times with PBA and then incubated for 30 min with Alexa Fluor 488 or Alexa Fluor 594 (1:100; Molecular Probes, CA, U.S.A.). MitoTracker Red 580 (Molecular Probes, CA, U.S.A.) was used for staining mitochondria. The coverslips were then washed three times and mounted onto glass slides with fluorescent mounting medium (DakoCytomation, U.K.). Images of cells were viewed using a Bio-Rad LaserSharp confocal microscope.

RESULTS

Cleavage of PKC δ After CVB3 Infection

Because the cleavage of PKC induced by apoptotic stimuli is a factor that contributes to PKC activation [15], we examined whether PKC δ was cleaved. As shown in Fig. 1A, PKC δ was cleaved from the full-length (FL) form to the catalytic fragment (CF) at 4, 6, and 8 h post-infection (pi), respectively, implying that PKC δ acts after 4, 6, and 8 h pi, respectively. Moreover, since various apoptotic stimuli induce caspase-dependent cleavage of PKC to generate the catalytically active fragment [17], we examined the cleavage of procaspase-3 after CVB3 infection. It was observed that procaspase-3 was cleaved at 6 h pi (Fig. 1A), suggesting that the cleavage of PKC may be related with caspase-3 activation. The viral capsid protein VP1 was first observed at 4 h pi (Fig. 1A), indicating that the cleavage of PKC δ occurs after the initiation of viral replication and protein expression.

Effects of CVB3 2A in Cleavage of PKC δ

Poliovirus 2A protein acts as a protease to induce apoptotic cell death [7]. Therefore, it is possible that CVB3 2A cleaves PKC δ. Thus, sequence encoding CVB3 2A was cloned into the pcDNA3.1 vector (Invitrogen, MD, U.S.A.) under the control of the CMV promoter, which was transfected

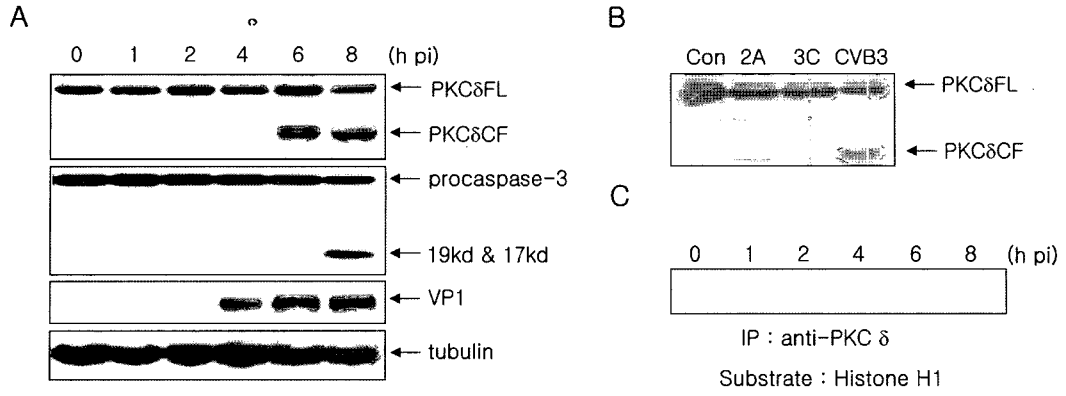

Fig. 1. Proteolytic activation of protein kinase C δ in CVB3-infected HeLa cells.
A. HeLa cells were infected with CVB3 (moi=20) and harvested at 0–8 h pi. Lysates were separated by SDS-PAGE, and immunoblotted using antibodies to PKC δ, caspase-3, and VP1. FL designates the full-length PKC, and CF designates the catalytic fragment. The active catalytic form of procaspase-3 is indicated by 19 kDa and 17 kDa. Expression of tubulin was used as an internal control. **B.** HeLa cells were transfected with pcDNA3.1 plasmid containing viral 2A or 3C genes or mock-transfected with empty pcDNA3.1 plasmid (Con). Lysates of CVB3-infected cells were used as a positive control (CVB3). **C.** At the indicated pi times, HeLa cells were harvested and lysate proteins were immunoprecipitated with anti-PKC δ antibody. Histone H1 was used as a substrate for the general kinase assay. Kinase activity was detected by autoradiography. Phosphorylation of histone H1 was increased from 6 h pi in lysates immunoprecipitated with anti-PKC δ antibody.

into HeLa cells for 30 h. Indeed, PKC δ was cleaved to catalytic fragments in CVB3 2A-transfected HeLa cells (Fig. 1B). Although CVB3 3C is also a protease like 2A, it did not cleave PKC δ (Fig. 1B). These results suggest that PKC δ is cleaved by viral protein 2A.

Activation of PKC δ After CVB3 Infection

To test whether PKC δ cleavage is associated with their activation, we performed the kinase assay. Interestingly, PKC δ was activated at 6 and 8 h pi (Fig. 1C). These data together indicate that PKC δ was cleaved and activated by CVB3 infection. Therefore, it can be inferred that the cleavage of PKC δ may be related with PKC δ activation in CVB3 infection.

Translocation of PKC δ after CVB3 Infection

One of the factors that may contribute to the distinct effects of PKC δ on cell apoptosis is the differential pattern of PKC translocation in response to various apoptotic stimuli [4]: Depending on the kind of stimuli, PKC δ is translocated to the plasma membrane, mitochondria, Golgi, ER, or nucleus [4]. Sindbis virus infection induces the translocation of PKC δ to the ER [26]. Here, we identified the translocation of PKC δ into the nuclei of HeLa cells after CVB3 infection, by using confocal microscopy and Western blot analysis (Fig. 2 and Fig. 3). HeLa cells on coverslips infected with CVB3 (moi=20) were fixed and incubated with anti-PKC δ antibody and then stained with FITC-conjugated goat anti-rabbit antibody (Abchem, U.K.) and DAPI. Nuclei were detected with DAPI staining, and the merged images clearly showed that PKC δ was localized to the nucleus after CVB3 infection (Fig. 2). PKC δ translocation into the nucleus was observed at 4 h pi, and after 8 h pi, PKC δ was dispersed from the nucleus as a consequence of the increased diffusion limits of the nuclear pores caused by apoptotic cell death (Fig. 2) [22]. To confirm the translocation of PKC δ to the nucleus, we separated the cytosolic and nuclear fractions of HeLa cells infected with CVB3 (moi=20) at the indicated times. Figure 3 shows that the full-length (FL) form of PKC δ in the cytosol was reduced, whereas the cleaved catalytic fragment (CF) of PKC δ was increased in the nucleus, indicating that the cleaved CF of PKC δ moved from the cytosol into the nucleus. According to Fig. 4, PKC δ after CVB3 infection was translocated into the nucleus via the mitochondria (Fig. 4, yellow region is colocalized with PKC δ and mitochondria at 1 h pi). Such translocation of PKC δ may lead to different cellular effects owing to the phosphorylation of specific substrates or to the association of PKC δ with distinct proteins present at specific locations [4]. However, the detailed function and mechanism of translocated PKC δ in the nucleus are unclear and require further investigation.

Role of PKC δ in CVB3 Infection

Because CVB3 infection induced the cleavage, activation, and translocation of PKC δ, we explored the role of PKC δ in HeLa cells infected with CVB3. We used PKC δ dominant-negative (DN) mutants, the kinase activity of which is inactivated by amino acid substitutions (PKC δ DN, K376R). The DN mutant of PKC was kindly supplied by Dr. J. W. Soh [Inha University, Department of Chemistry, Korea; 23]. First, we examined the expression levels of the wild-type (WT) and DN forms of PKC δ fused to HA by immunoblotting with anti-HA antibody (Upstate, MA, U.S.A.) to exclude the possibility of differential expression

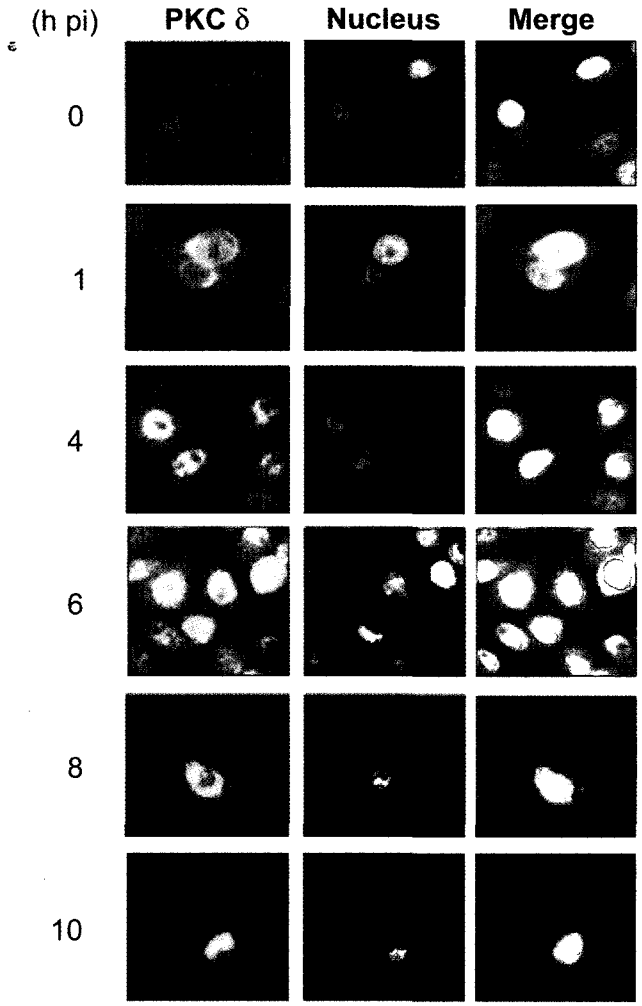

Fig. 2. Subcellular localization of PKC δ in HeLa cells infected with CVB3 by confocal microscopy.
At each indicated time point, cells were fixed, probed with anti-PKC δ antibody, and stained with DAPI for nucleus. Confocal microscopy images were merged.

of these plasmids in the transfected cells. There was no significant difference in the expression levels (Fig. 5A). As

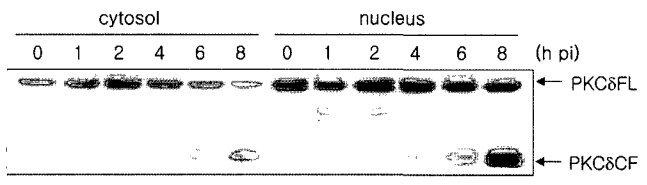

Fig. 3. Translocation of PKC δ to the nucleus in HeLa cells infected with CVB3.
HeLa cells were infected with CVB3 (moi=20). At indicated time, cells were lysed and cytosolic and nuclear fractions were prepared. Each fraction was subjected to immunoblotting analysis using anti-PKC δ antibody. Right arrows indicate PKC δ FL (full length) and PKC δ CF (catalytic fragment).

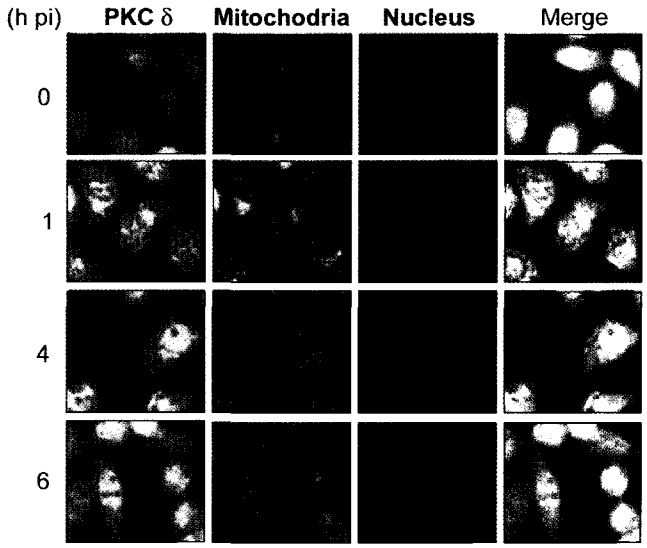

Fig. 4. Translocation of PKC δ via mitochondria in HeLa cells infected with CVB3.
At each indicated time point, cells were fixed, probed with anti-PKC δ antibody, and MitoTracker Red 580 was used for staining mitochondria, and DAPI for nucleus. Confocal microscopy images were merged. The yellow region in merge showed colocalization of green and red, and the light-blue region showed colocalization of green and blue.

a next experiment, we transfected HeLa cells with WT and DN plasmids encoding PKC δ for 30 h and then infected with CVB3 (moi=20). As a control, HeLa cells were transfected with empty pcDNA3.1 (mock-transfected). At 10 h pi, apoptotic cell death was detected by FACS analysis after the cells were stained with annexin V-FITC (Molecular Probes, CA, U.S.A.). PKC δ WT reduced apoptotic cell death by about 50%, compared with the controls; that is mock-transfected and CVB3-infected HeLa cells (21.44±0.25% of control cells were apoptotic, whereas 10.17±0.14% of PKC δ WT-transfected cells were apoptotic) (Fig. 5B). However, PKC δ DN did not confer any protective effect against the apoptosis induced by CVB3 infection. These results indicate that PKC δ plays an anti-apoptotic role in HeLa cells infected with CVB3.

DISCUSSION

Although the apoptosis-associated factors involved in CVB3 infection have already been reported [5], the upstream signal transduction factors of apoptosis induced by CVB3 infection are still unclear. One of the candidates is the PKCs, because they have been implicated as important regulators of cell apoptosis [8, 20]. PKC δ and ε show contradictory effects on apoptosis in heart disease: PKC δ is pro-apoptotic and PKC ε is anti-apoptotic [1, 21]. In the present study, PKC δ was cleaved after CVB3 infection (Fig. 1A) and was activated in infected HeLa cells (Fig. 1C).

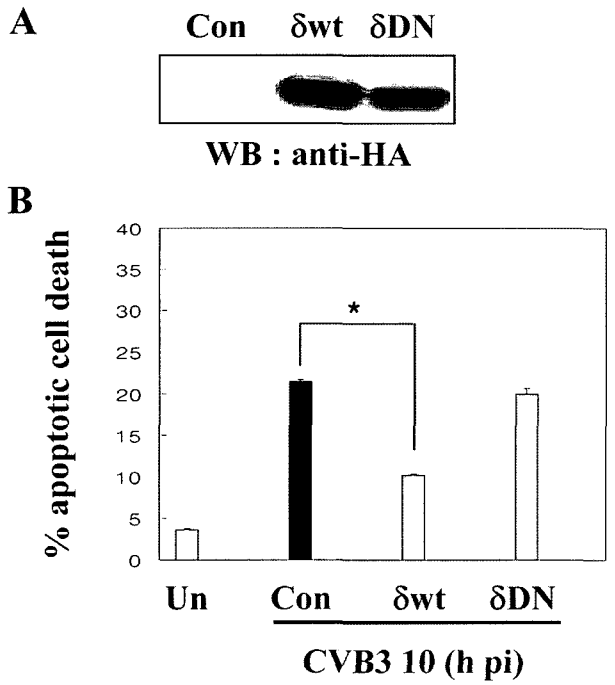

Fig. 5. Anti-apoptotic effect of PKC δ WT in CVB3-infected HeLa cells.

A. HeLa cells were transfected with plasmids PKC δ WT and PKC δ DN tagged with HA. After transfection for 30 h, cells were lysed and the lysates were immunoblotted using anti-HA antibody. Mock-transfected pcDNA3.1 was the control (con). **B.** As described above (Fig. 5A), cells were transfected with each plasmid for 30 h. After transfection, cells were infected with CVB3 (moi=20). After 10 h pi, samples were analyzed by FACS. Apoptotic cell death was calculated by staining cells with annexin V-FITC after CVB3 infection at 10 h pi. The results represent means± standard deviation of triplicate measurements in each of three experiments. *, $p<0.001$ as compared with control infected cells (con).

We also examined the cleavage and activation of PKC ε after CVB3 infection. Although PKC ε was cleaved after CVB3 infection, it was not activated (data not shown). Therefore, we focused our further study on PKC δ. PKC δ was translocated to the nucleus via mitochondria after CVB3 infection (Figs. 2, 3, and 4). We also found a novel role for PKC δ in CVB3 infection, in that it exerts a protective effect against the apoptotic cell death induced by CVB3 infection (Fig. 5B). An interesting finding in this study is that PKC δ has an anti-apoptotic role, which is in contrast to its predominant activity as a pro-apoptotic kinase. However, our results are consistent with previously reported data demonstrating that PKC δ exerts anti-apoptotic effects in Sindbis virus infection [26]. These opposite roles of PKC δ may be explained by its differential translocation, which leads to the phosphorylation of different substrates and the phosphorylation of different tyrosine residues of PKC δ, which in turn can alter the affinity of PKC δ for its different substrates [9, 26]. Therefore, the role of PKC δ may be to protect cells against the apoptotic cell death induced by CVB3 infection. However, this protection may be exerted at an early stage (less than 10 h pi) of CVB3-induced apoptosis, because it does not act in later stages (24 h pi) of CVB3-induced cell death (data not shown).

Recently, it was reported that the phosphatidylinositol 3-kinase/protein kinase B/Akt pathway plays a critical role in CVB3 replication [6]. These recent data and the role of PKC δ demonstrated in this study clarify the mechanism of apoptotic cell death induced by CVB3 infection and ultimately the pathophysiological mechanism of myocarditis and dilated cardiomyopathy caused by CVB3 infection.

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