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The η isoform of protein kinase C inhibits UV-induced activation of caspase-3 in normal human keratinocytes

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Abstract

Protein kinase C (PKC) fulfills a central role in the decision of cell fate in keratinocytes. Both PKCδ and PKCη induce growth inhibition and differentiation of normal human keratinocytes (NHK). Here we show that PKCδ and PKCη play opposite roles in UVB-induced apoptosis in NHK. PKCδ enhanced UVB-induced caspase-3 activity, while overexpression of PKCη reduced it. In keeping with these observations, the dominant negative mutant of PKCδ significantly inhibited the activation of caspase-3, whereas dominant negative PKCη increased it in a dose (MOI)-dependent manner. Unlike PKCδ, cleavage and translocation to mitochondria of PKCη were not observed, resulting in no detection of cytochorome *c* release. Furthermore, UV-induced activation of p38 MAP kinase, which suppressed the caspase-3 activity in NHK, was blocked by dominant negative PKCη. These findings suggest that PKCη negatively regulates UV-induced apoptosis through its localization, resistance to cleavage, and the p38 MAPK pathway.

Keywords: Keratinocytes; Apoptosis; Ultraviolet radiation; Protein kinase C

Apoptosis is a regulated cell death essential for development of tissues and maintaining the homeostasis of multicellular organisms. Keratinocytes defend the skin against environmental stresses such as chemical stimulation and UV exposure. UV irradiation from the sun is a major cause of gene mutation, subsequently resulting in the formation of multiple types of skin cancers. Apoptosis of keratinocytes is an important mechanism for protecting skin from carcinogenesis. A better understanding of how UV induced-apoptosis is regulated in normal keratinocytes is essential for clarification of the mechanisms of skin carcinogenesis.

Protein kinase C (PKC) is a serine/threonine kinase which regulates a wide variety of cellular functions including proliferation, differentiation, and apoptosis [1]. PKC consists of at least 11 isoforms which are classified into classical (α, β1, β2, and γ), novel (δ, ε, η, θ, and μ), and atypical (ζ, λ/ι) subfamilies based upon their molecular structure and co-factor requirements [1]. Among these isoforms, PKCδ plays a major role in the progression of apoptosis in a variety of cells. Phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), induces translocation of PKCδ from cytosol to mitochondria and subsequently causes the release of cytochrome *c* [2]. PKCδ is cleaved at the third variable region by many genotoxic stimuli including UV radiation, generating a 42 kDa constitutively active fragment, which in turn evokes apoptosis [3,4]. Overexpression of the active fragment of PKCδ disrupted the mitochondrial membrane potential, released cytochrome *c*, and induced apoptosis in human keratinocytes [5]. In several types of cells, PKCε, θ, and ζ were involved in the process of apoptosis [6–9]. PKCζ and λ/ι were cleaved by caspase and inhibited UV-induced apoptosis in NIH3T3 and COS1 cells [9,10]. PKCε is translocated to cell membranes during UV-induced apoptosis and involved in the activation of ERK and JNK MAP kinases in mouse

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epidermal JB6 cells [11]. In normal human keratinocytes, however, clear evidence of whether PKC isoforms other than PKCδ regulate the UV-induced apoptosis are not indicated.

In keratinocytes, five PKC isoforms are expressed, i.e., PKCα, δ, ε, η, and ζ. We have previously reported that PKCδ and η regulate cell growth and differentiation of keratinocytes [12]: both enzymes induce cell-cycle arrest at the G1 phase and the activation of transglutaminase 1 [12]. In addition, Efimova and Eckert [13] have shown that the expression of involcrin mRNA, a differentiation marker of keratinocytes, is increased by both PKCδ and PKCη. However, PKCδ is located in whole cell layers of the epidermis, whereas PKCη is restricted to differentiated layers [33]. Moreover, in our earlier study, we pointed out the morphological difference between PKCδ- and PKCη-overexpressing keratinocytes, that is, PKCδ induced a spindle-shaped NHK and PKCη induced a squamous phenotype [12]. These findings suggest the possibility that PKCδ and PKCη may have some shared cellular functions in keratinocytes.

In the current study, we investigated the effects of all the PKC isoforms expressed in keratinocytes on UVB-induced caspase-3 activity. Our observation demonstrated that PKCδ activates caspase-3, while PKCη reduces its activity.

Materials and methods

Cell culture, UV treatment, and reagents. Normal human keratinocytes (NHK) were purchased from Clonetics (San Diego, CA) and cultured in keratinocyte growth medium (KGM; Clonetics). For expansion, the cells were trypsinized with 0.025% trypsin and 0.02% EDTA and plated in KGM. Apoptosis of NHK was induced by UV lamp (UVP, Upland, CA). The output wavelength of the bulbs was 65% UVB, 20% UVA, and 15% UVC. Twenty to 40 mJ/cm^2 UVB was routinely used for induction of apoptosis. Anti-PKCα, anti-PKCδ, anti-PKCε, anti-PKCη, and anti-PKCζ antibodies, which recognize C-terminus of these PKC, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cytochrome *c* antibody was purchased from PharMingen (San Diego, CA). Phospho-p38 MAPK antibody, which specifically recognizes the phosphorylated form of its kinase, and anti-p38 antibody were from New England Biolabs (Beverly, MA). SB203580, p38 MAPK inhibitor, was from Calbiochem (La Jolla, CA).

Adenovirus-mediated gene transfer. Replication-deficient Ad5-type adenovirus vectors containing the cDNA of both wild and kinase negative mutants of rabbit PKCα, mouse PKCδ, rabbit PKCε, mouse PKCη, and mouse PKCζ were constructed as described previously [12,15]. In brief, cDNA was inserted into pAxCAwt cosmid, generating a recombinant adenovirus vector. Purification and titration of the adenovirus were performed as previously described [12]. Kinase negative mutants of PKC were generated by substitution of arginine or alanine for lysine at the ATP binding site of PKC [12,16,17].

Cell fractionation and immunoblotting. Whole cell lysates were prepared by lysing the floating and attached cells in 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 20 mM NaF, 50 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Lysates were centrifuged for 10 min at 4 °C and equal amounts of protein from the supernatants were used for SDS–PAGE and immunoblotting. For cell fractionation, floating and attached cells were washed with cold phosphate-buffered saline (PBS) and lysed with PKC lysis buffer (20 mM Hepes, pH 7.4, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 50 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). The lysates were gently homogenized for 30 strokes in a glass homogenizer. Unlysed cells and nuclei were removed by centrifugation for 10 min at 750*g*. The supernatants were centrifuged at 10,000*g* for 30 min at 4 °C. Pellets were suspended in the lysis buffer and taken as the mitochondria-containing fraction. The supernatants were spun at 100,000*g* for 1 h and were taken as the cytosol fraction. Fifteen micrograms of proteins from both fractions were used for immunoblotting. The proteins were run on SDS–polyacrylamide gels and transferred to nitrocellulose. The membranes were blocked with 5% skim milk or 3% BSA, stained with primary antibodies and HRP-conjugated second antibodies, respectively. Proteins were detected using the Western Lightning Chemiluminescence Reagent Plus (Perkin–Elmer Life Sciences).

Caspase-3 activity. For the measurement of caspase-3 activity, Caspase-3 Colorimetric Assay Kit (R&D Systems, Minneapolis, MN) was used. Following UV-treatment, the attached cells were washed twice with a cold PBS and lysed in a caspase lysis buffer, while floating cells were collected by centrifugation and then added into the same lysis buffer. The lysates were spun at 10,000*g* for 5 min at 4 °C and the supernatants were collected for the assay. Equal amounts of proteins (100 μg) were reacted in a buffer containing 0.2 mM DEVD-pNA substrate and 5 mM DTT at 37 °C for 1–4 h, and then absorbance at 405 nm was measured. Caspase-3 activity was represented as a relative increase to the non-UV-treated cells.

Results

UV-induced caspase-3 activity in PKCδ- and PKCη-overexpressing NHK

Throughout the present study, we used the adenovirus vectors expressing the wild and dominant negative mutants of α, δ, ε, η, and ζ isoforms of PKC. The adenovirus expressing β-galactosidase gene (LacZ) was used as a negative control. Proteins of all PKC isoforms were strongly expressed in normal human keratinocytes when infected with these PKC adenovirus vectors (Fig. 1).

The activity of caspase-3, an indicator of apoptosis, was measured by using the cleavage of a specific colorimetric peptide substrate. A 4-fold increase of caspase-3 activity was observed in LacZ-infected keratinocytes after UVB irradiation (Fig. 2A). When wild type PKCδ was overexpressed, a further increase of caspase-3 activity was observed in UVB-exposed cells. In contrast, overexpression of PKCη decreased the UV-induced caspase-3 activity to approximately 75% of that of the LacZ-expressing cells (Fig. 2A). These contrastive effects between PKCδ and PKCη were more apparent in the experiment shown in Fig. 2B. In a dose (MOI)-dependent fashion, PKCδ enhanced the caspase-3 activity and PKCη reduced it. The other three PKC isoforms, PKCα, PKCε, and PKCζ, had no effect on caspase-3 activity (Fig. 2A).

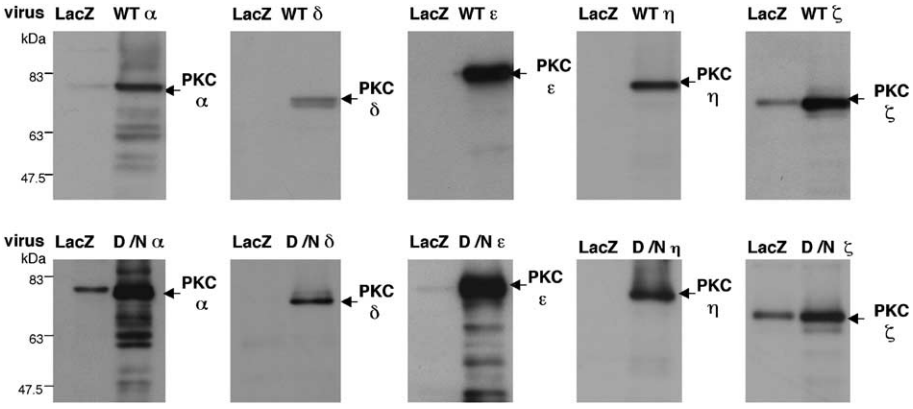

Fig. 1. Expression of PKC isoforms in adenovirus-infected normal human keratinocytes. Normal human keratinocytes were infected with adenovirus vectors of lacZ, wild type (WT), and dominant negative type (D/N) of PKCα, PKCδ, PKCε, PKCη, and PKCζ, and whole cell lysates were collected at 40 h after infection and subjected to Western blotting.

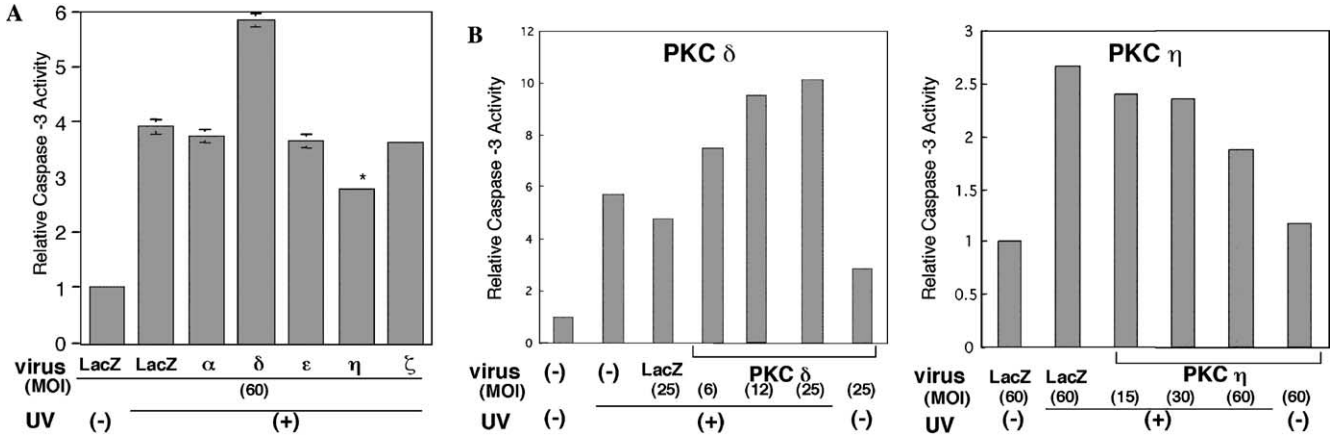

Fig. 2. PKCη inhibits UV-induced caspase-3 activation in normal human keratinocytes. (A) Effects of five PKC isoforms on caspase-3 activity. NHK were infected with adenovirus vector of LacZ (LacZ) or each wild type PKC isoform (α: PKCα, δ: PKCδ, ε: PKCε, η: PKCη, and ζ: PKCζ) at MOI = 60 and were exposed to UVB (40 mJ/cm^2) after 24 h of infection. After 16 h, caspase-3 activity was assayed using colorimetric peptide substrate selective for caspase-3. Values represent means ± SD of triplicate determinations and are shown as a relative ratio to the value for lacZ-infected cells without UV stimulation. Asterisks indicate the significant difference from LacZ-infected cells (*, $P < 0.05$). (B) MOI-depending effects of PKCδ and PKCη on caspase-3 activity. Keratinocytes were infected with the indicated MOI of PKCδ and PKCη adenoviruses for 24 h, and were exposed to UVB (40 mJ/cm^2). Caspase-3 activity was assayed after 16 h of UV irradiation.

Increase of caspase-3 activity by dominant negative PKCη

We further examined the effects of PKC isoforms on UVB-induced apoptosis by using the dominant negative mutants of PKC. As shown in Fig. 3A, dominant negative PKCδ caused significant reduction of the caspase-3 activity induced by UV. In contrast, dominant negative PKCη enhanced caspase-3 activity more than two times (Fig. 3A). Its activity was increased by the dominant negative PKCη in a dose (MOI) -depending manner (Fig. 3B). However, dominant negative mutants of PKCα, ε, and ζ did not influence the UVB-induced caspase-3 activity (Fig. 3C). These results indicate that PKCδ regulates UVB-induced caspase-3 activity positively, while PKCη regulates it negatively.

Translocation to mitochondria and cleavage of PKCδ and PKCη

To clarify how PKCη inhibits UVB-induced apoptosis, we first determined the cleavage of PKCδ and PKCη. Previous studies have demonstrated that the δ, ε, and θ isoforms of PKC are cleaved by caspase during apoptosis, resulting in the generation of active catalytic fragments [4,7,9]. As shown in Fig. 4A, the cleaved product of PKCδ was generated by UV irradiation. However, only a faint band was found in PKCη-overexpressing cells under the same conditions (Fig. 4A, lower panel). Next, we investigated the translocation of PKCη to mitochondria from cytosol after UVB irradiation. Fig. 4B shows that UV irradiation increased the

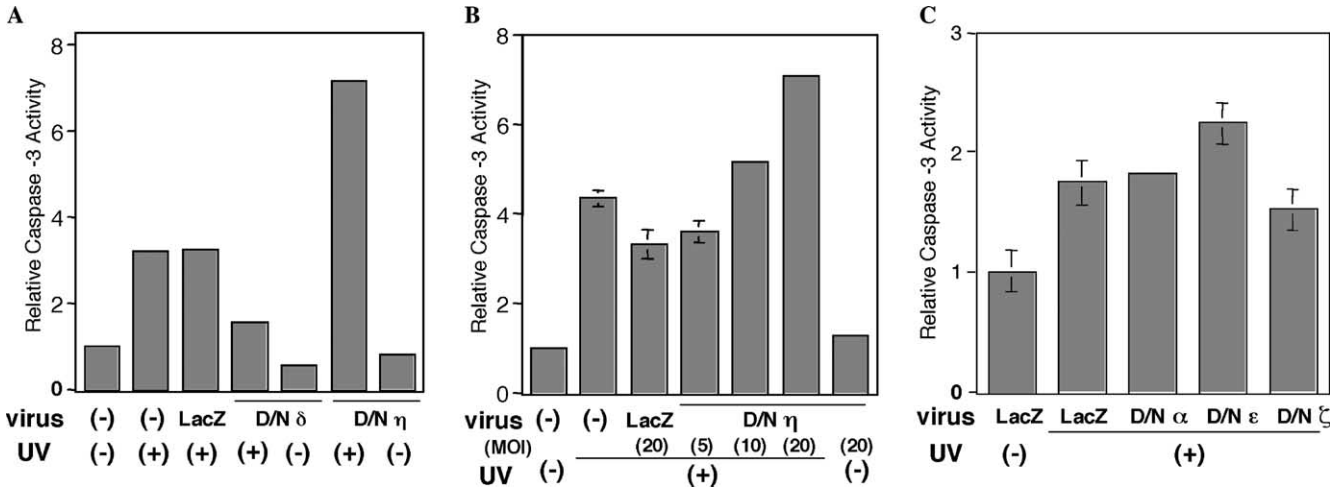

Fig. 3. Dominant negative PKCη enhances UV-induced caspase-3 activity in normal human keratinocytes. (A) Effects of dominant negative PKCδ and η on UV-induced caspase-3 activity. NHK infected with adenovirus vectors of lacZ (lacZ) or dominant negative mutant (D/N) of PKCδ and η were irradiated with 40 mJ/cm^2 UVB. (B) Dose-depending increase of UV-induced caspase-3 activity by dominant negative PKCη. Cells were infected with the indicated MOI of D/N η adenovirus vector and irradiated with UVB. (C) Effects of three dominant negative PKC isoforms on UV-induced caspase-3 activity. NHK infected with dominant negative mutants (D/N) of PKCα, ε, and ζ were irradiated with UVB. In all experiments (A, B, and C), cells were infected at MOI = 20 for 24 h, and were irradiated with 40 mJ/cm^2 UVB. The caspase-3 activity was determined after 16 h of UV irradiation. Values represent means ± SD of triplicate determinations and are shown as a relative ratio to non-infected (A, B) or lacZ-infected (C) cells without UV stimulation.

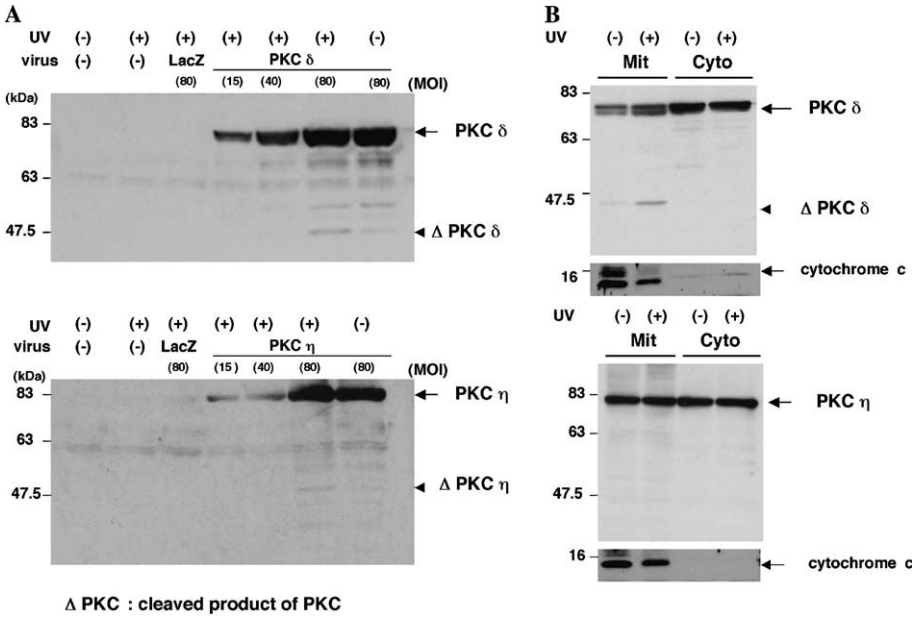

Fig. 4. Cleavage of PKCδ and η by UV irradiation in normal human keratinocytes. (A) Cleavage product of PKC in whole cell lysates. Cells were infected with adenovirus vector of PKCδ or PKCη for 24 h and were exposed to UVB (40 mJ/cm^2), and whole cell lysates were collected after 16 h and subjected to Western blotting by using a specific antibody against the C terminal domain of PKCη or PKCδ. Anti-PKCδ antibody was specifically recognized as exogenous mouse PKCδ. (B) Cells were infected with adenovirus vector of PKCδ (top) or PKCη (bottom) at MOI = 20 for 24 h and were exposed to UVB (40 mJ/cm^2). Cells were lysed after 16 h and fractionated into mitochondrial (Mit) and cytosolic fractions (Cyto) as described in Materials and methods. Western blots were performed with the indicated antibodies. The arrowhead in the upper panel indicates the position of the 42 kDa of PKCδ cleaved product, and the arrowhead in the bottom panel indicates the 50 kDa of PKCη cleaved product.

mitochondrial PKCδ and concomitantly decreased the cytoplasmic PKCδ. A 42 kDa of cleavage product of PKCδ was also detected only in the mitochondrial fraction. However, PKCη was not translocated to mitochondria and the cleavage product of PKCη was not detected in UV-exposed cells (Fig. 4B). We failed to observe translocation to mitochondria of PKCη by immunofluorescence analysis (data not shown). More-

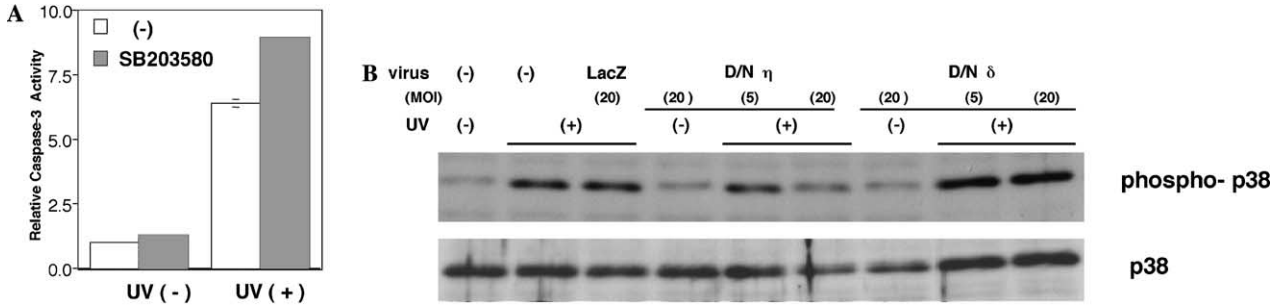

Fig. 5. Effect of dominant negative PKC on p38 MAPK activity. (A) Enhancement of UV-induced caspase-3 activity by p38 MAPK inhibitor. NHK were incubated for 4 h with 10 μM SB203580, or vehicle alone (−) (0.05% DMSO) before UVB-irradiation (40 mJ/cm^2). The inhibitor was withdrawn during UV irradiation and then added back. The caspase-3 activity was determined at 24 h after irradiation. (B) Inhibition of p38 MAPK phosphorylation by dominant negative PKCη. Cells were infected with dominant negative PKCη and δ adenovirus vector at the indicated MOI, and the cells were starved with keratinocyte basal medium lacking the growth supplements for 48 h. Then cells were exposed to 40 mJ/cm^2 UVB and whole cell lysates were collected at 30 min after UV irradiation. p38 and phosphorylated p38 proteins were detected by Western blotting using the specific antibodies.

over, release of cytochrome *c* into cytosol by UV exposure, which was detectable in PKCδ-overexpressing cells, was not found in PKCη-overexpressing keratinocytes (Fig. 4B). In addition, there were no mitochondrial translocation and UVB-induced cleavage of PKCα, ε, and ζ (data not shown). These findings suggest that no mitochondrial translocation and resistance to cleavage of PKCη result in no increase of caspase-3 activity in PKCη-overexpressing cells.

Phosphorylation of p38 MAPK in the dominant negative PKCδ- and PKCη-overexpressing keratinocytes

To further explore the mechanism of how PKCη inhibits caspase-3 activity, we examined the involvement of PKCη in the activity of p38 mitogen-activated protein kinase (MAPK). MAPK family, especially p38 MAPK, plays a crucial role in transducing signals induced by stress-stimuli including UV irradiation [18]. After UV exposure, p38 MAPK is rapidly phosphorylated and sustained its phosphorylated state [19]. Fig. 5A shows that inhibition of p38 MAPK by SB203580, a p38 specific inhibitor, enhanced UVB-induced caspase-3 activity, indicating that p38 negatively regulates the caspase-3 activity in normal keratinocytes. We next examined the effects of dominant negative PKCη on the activity of p38. As shown in Fig. 5B, dominant negative PKCη diminished the UV-induced phosphorylation of p38 in a dose-dependent manner, while dominant negative PKCδ slightly increased the p38-phosphorylation. These findings suggest that PKCη interferes with UV-induced caspase-3 activation through the p38 MAPK pathway.

Discussion

In the present report, we demonstrated that PKCδ increased the activation of caspase-3, while PKCη reduced it in normal human keratinocytes (Figs. 2 and 3). Consistent with this finding, cleavage and translocation to mitochondria, which are important events in the PKC-mediated apoptosis pathway, were not observed in PKCη-expressing cells during apoptosis (Fig. 4). Our previous study showed that both PKCδ and PKCη exhibit the same abilities in the induction of keratinocyte differentiation [12,14]. However, PKCδ and PKCη have diverse properties in the regulation of UV-induced apoptosis.

Several reports have shown the implication of PKC in UV-induced apoptosis. In NIH3T3 and HeLa cells, PKCζ inhibited UV-induced apoptosis through the modulation of Bcl-2 [9,10]. PKCε was translocated to membranes and activated by UV in JB6 epidermal cell lines [11]. However, our present data show that PKCε and ζ exhibited no influence on caspase-3 activity (Figs. 2A and 3C) and DNA fragmentation (data not shown). Denning et al. [4] also reported no translocation of PKCε to the membrane fraction after UV exposure in human keratinocytes. These results found in keratinocytes suggest the existence of keratinocyte-specific mechanisms to regulate apoptosis via PKC. In addition, keratinocytes are the main target of UV damage. Thus, it is worth knowing the UVB-induced apoptotic signaling pathways in normal keratinocytes.

In several types of cells including keratinocytes, PKCδ is known to cleave and activate during the process of apoptosis [5,6,21]. PKCδ is cleaved at DMQD^{330}/N mediated by caspase-3 [3]. PKCθ is also proteolyzed at a DEVD^{354}/K site in the hinge region by DNA damaging agents [8]. Overexpression of these cleaved, active fragments of PKCs led to activation of caspase-3 and DNA fragmentation [3,5,8]. As for PKCη, cleavage and its activation are likely depending on cell types. In keratinocytes, as shown in Fig. 4, there is no or little evidence of cleavage of PKCη. This can be explained by the lack of proper caspase-3 recognition

site (DEXD/N) in its hinge region. However, in pre- and pro-B cells, PKCη was efficiently proteolyzed in the hinge region after exposure to UVB, generating 50 kDa of truncated, kinase active form [22]. One possible explanation is that PKCη is proteolyzed in B cells by some other unidentified protease than caspase-3, and keratinocytes have either no expression of such a protease or no upstream pathway activating this protease.

PKCδ is shown to translocate to mitochondria by UVB and TPA stimulation (Fig. 4B) [4,23]. Ectopically expressed truncated active fragment of PKCδ is also localized in mitochondria, leading to the activation of caspase-3 [5]. In the case of PKCη, there was no translocation to mitochondria after UV irradiation. Previous studies have demonstrated that PKCη is mainly localized at rough endoplasmic reticulum [24] and its localization remains unchanged after treatment of TPA and bryostatin [25,26]. Lack of mitochondrial translocation of PKCη may be related to its unique localization in keratinocytes, though the definite mechanisms have yet to be investigated.

UV irradiation leads to activation of the MAPK superfamily, ERKs, JNKs, and p38 MAPK [27–29]. p38 MAPK is particularly involved in the regulation of stress response and apoptosis [30,31]. Generally, activation of p38 promotes the apoptosis in various types of cells [30]. In a human immortalized keratinocyte cell line HaCaT, inhibition of p38 MAPK by SB203580 suppressed both caspase-3 activity and apoptosis [19,32]. However, in normal keratinocytes, inhibition of p38 MAPK by SB203580 enhanced caspase-3 activity, indicating that p38 MAPK functions as a negative modulator in UV-induced apoptosis in normal keratinocytes (Fig. 5A). This finding is consistent with the report of Chouinard et al. [20]. They showed that SB203580 and dominant negative p38 increased UVB-induced apoptosis in normal keratinocytes. Furthermore, we found that dominant negative PKCη suppressed p38 phosphorylation (Fig. 5B) and increased UV-induced caspase-3 activity (Figs. 3A and B). Taken together, it may be supposed that PKCη blocks caspase-3 activity through p38 MAPK. The mechanism of how PKCη modulates the p38 activity remains to be determined. Our preliminary data showed that dominant negative PKCη reduced the activity of MKK3 MAPK. PKCη may negatively regulate the upstream pathway of MKK3 MAPK kinase.

What is the physiological significance of the fact that PKCη inhibits UV-induced apoptosis of keratinocytes? PKCη is highly expressed in the differentiating and differentiated suprabasal layers of the epidermis [33]. It is supposed that PKCη prevents the excess or unnecessary cell death in the differentiated keratinocytes to establish a barrier to insults and environmental stress. Proper regulation of apoptosis of keratinocytes is crucial for the protection of the organism.

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