Parthenolide sensitizes ultraviolet (UV)-B-induced apoptosis via protein kinase C-dependent pathways

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Parthenolide (PN) is the principal sesquiterpene lactone in feverfew (Tanacetum parthenium) with proven anti-inflammatory properties. We have previously reported that PN possesses strong anticancer activity in ultraviolet B (UVB)-induced skin cancer in SKH-1 hairless mice. In order to further understand the mechanism(s) involved in the anticancer activity of PN, we investigated the role of protein kinase C (PKC) in the sensitization activity of PN on UVB-induced apoptosis. Several subtypes of PKC have been reported to be involved in UVB-inhibited signaling cascade with both pro- and anti-apoptotic activities. Here we focused on two isoforms of PKC: novel PKCζ and atypical PKCδ. In JB6 murine epidermal cells, UVB induces the membrane translocations of both PKCs, and PN pre-treatment enhances the membrane translocation of PKCδ, but inhibits the translocation of PKCζ. Similar results were also detected when the activities of these PKCs were tested with the PKC kinase assay. Moreover, pre-treatment with a specific PKCζ inhibitor, rottlerin, completely diminishes the sensitization effect of PN on UVB-induced apoptosis. When cells were transiently transfected with dominant negative PKCζ or wild-type PKCζ, the sensitization effect of PN on UVB-induced apoptosis was also drastically reduced. Further mechanistic study revealed that PKCζ, but not PKCδ, is required for UVB-induced p38 MAPK activation and PN is likely to act through PKCζ to suppress p38 activation in UVB-treated JB6 cells. In conclusion, we demonstrated that PN sensitizes UVB-induced apoptosis via PKC-dependent pathways.

Abbreviations: AP-1, activator protein-1; DAG, diacylglycerol; DN, dominant negative; MAPKs, mitogen activated protein kinases; NF-κB, nuclear transcription factor-kappa B; NF-AT, nuclear factor of activated T cells; PKC, protein kinase C; PN, parthenolide; UVB, ultraviolet B light.

Introduction

Feverfew (Tanacetum parthenium) has been used as a herbal plant for centuries in Europe, with known anti-microbial and anti-inflammatory properties (1,2). Parthenolide (PN), a sesquiterpene lactone, is one of the principal bioactive components of this plant. It is believed that the bioactivity of PN is mediated via the highly electrophilic α-methylene-γ-lactone ring and an epoxide residue which are capable of interacting rapidly with nucleophilic sites of biological molecules (3,4). PN is a potent inhibitor of DNA synthesis and cell proliferation in a number of cancer cell lines (5–7). In addition, PN has been reported to induce apoptosis via caspase activation and mitochondria dysfunction (8), disruption in intracellular thiols and calcium equilibrium (9), as well as activation of pro-apoptotic Bcl-2 family proteins (10). On the other hand, PN also has been shown to sensitize cells to apoptosis induced by various stimuli such as ultraviolet B (UVB), tumor necrosis factor-α (TNF-α) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), presumably through its action on activator protein-1 (AP-1) signaling pathway (11–13). The in vivo anticancer activity of PN has been studied recently. For instance, we have demonstrated that PN possesses a strong chemopreventive property against UVB-induced skin cancer in SKH-1 hairless mice (12), whereas Sweeney et al. (14) revealed that PN in combination with docetaxel is capable of reducing metastasis and improving survival in the xenograft model of breast cancer.

The electromagnetic spectrum of ultraviolet (UV) can be grouped into UVA (320–400 nm), UVB (280–320 nm) and UVC (200–280 nm). UVB exposure is the main etiological factor for non-melanoma skin cancer in human (15). The mechanism(s) of UVB-induced skin cancer have not been fully understood. Several transcriptional factors including AP-1, nuclear transcription factor-kappa B (NF-κB), nuclear factor of activated T cells (NF-AT), and signal transducers and activators of transcription have been linked to the tumour-promoting ability of UVB (16–19).

Protein kinase C (PKC) is a group of serine/threonine kinases that regulate many cellular functions such as proliferation, differentiation, transformation, survival and apoptosis (20). PKC can be classified into three groups based on the co-factors required for activation: (i) the Ca²⁺ and diacylglycerol (DAG)-dependent classical or conventional PKC that consists of isoforms α, β1, β2 and γ; (ii) the DAG-dependent, Ca²⁺-independent novel PKC that consist of δ, η, ε and θ; and (iii) the DAG- and Ca²⁺-independent atypical PKC that consist of ι/λ and ζ. The consequences of PKC activation by UVB is rather cell-type specific and could lead to inhibition on cell proliferation or even induction of apoptosis. Among all, PKCζ seems to be the main PKC subtype with pro-apoptotic functions in response to various extracellular stimuli including UVB (21,22), whereas PKCζ has been shown to be anti-apoptotic in response to UV (23,24).

Our previous findings (12) showed that the suppression of mitogen activated protein kinase (MAPK) and AP-1 signaling cascade by PN contributes to its sensitization effect on UVB-induced apoptosis in JB6 cells. It is also known that certain subtypes of PKC regulate the MAPK/AP-1 pathway in UVB-treated cells (21,25). Hence, the main objective of this study is to explore the involvement of PKC to further understand the underlying mechanism(s) of the chemopreventive property of PN. Our data demonstrated here that PN selectively inhibits UVB-induced PKCζ activation and subsequent p38 MAPK activation, while further enhancing UVB-induced PKCζ activation.

Carcinogenesis vol.26 no.12 © Oxford University Press 2005; all rights reserved.
activity; both contribute to the sensitization effect of PN on UVB-induced apoptosis in murine epidermal JB6 cells.

Materials and methods

Chemicals and reagents

PN (97% pure) was purchased from Biomol (Plymouth Meeting, PA). Anti-HA, PKC-δ and ε polyclonal antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Secondary antibodies (horseradish peroxidase conjugated goat anti-rabbit IgG and goat anti-mouse IgG) and enhanced chemiluminescence substrate were from Pierce (Rockford, IL). γ-32P ATP was obtained from Perkin–Elmer (Boston, MA). PKC inhibitors GF109203X and rotterlin were purchased from Calbiochem (San Diego, CA). Other common chemicals were from Sigma–Aldrich (St Louis, MO).

Cell culture and UVB exposure

JB6 murine epidermal cells were cultured in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO2/air atmosphere as described previously (12). Cells were seeded in 60 mm cultural dishes and starved with MEM containing 0.5% FBS for 24 h before reaching 80% confluence. After pre-treatment with various reagents at designated conditions, cells were washed with PBS once and then exposed to UVB in fresh PBS. UVB was delivered through a band of FS24 lamps (Light Sources, Orange, CT) with spectral irradiance of 280–400 nm, 80% of which was in the UVB region (280–320 nm) with a peak at ~313 nm. The emitted UVB dose was quantified using a photothero-therapy radiometer (International Light, Newburyport, MA) equipped with IL SED 240 detector. Cells were returned to the incubator with the addition of the previous culture medium until the time of collection.

Transient transfection

Wild-type and dominant negative (DN) PKCδ and PKCζ plasmids were kindly provided by Dr J.W. Soh from Inha University, Incheon, Korea. DN-p38 and DN-p38β plasmids were gifts from Dr J.Han (Scirpis Research Institute, La Jolla, CA, USA). Cells were co-transfected with designated PKC plasmids and a transfection marker pDsRed (Clontech, Palo Alto, CA) using Lipo-fection AMINE reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. Cells were subjected to various treatments 24 h after transfection.

Detection of apoptotic cell death

Following the designated treatments, apoptotic cell death was quantified using DNA content analysis (sub-G1 cells) as described previously (26). In some experiments, cells were first transiently transfected with various PKC plasmids and pDsRed. Apoptotic cell death was then determined using DNA content analysis coupled with flow cytometry after 4′,6-diamidino-2-phenylindole (DAPI) staining. Briefly, cells were washed with phosphate buffered saline (PBS), fixed first with 0.5% para-formaldehyde and then with 70% ethanol. After staining with DAPI, 20 000 cells from each group were analyzed by flow cytometry using Becton Dickinson FACSVantage SE system (Franklin Lakes, NJ, 27). Only those transfected cells with expression of the red fluorescence protein were then gated for analyzing the percentage of sub-G1 cells using WinMDI 2.7 software (Scirpis Institute, La Jolla, CA).

PKC translocation assay

PKC translocation assay was performed based on a published method (21) with modifications. In brief, 15 min after UVB irradiation cells were washed once with ice-cold PBS and then sonicated in homogenization buffer with a peak at ~313 nm. The emitted UVB dose was quantified using a photothero-therapy radiometer (International Light, Newburyport, MA) equipped with IL SED 240 detector. Cells were returned to the incubator with the addition of the previous culture medium until the time of collection.

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PKC translocation assay

PKC translocation assay was performed based on a published method (21) with modifications. In brief, 15 min after UVB irradiation cells were washed once with ice-cold PBS and then sonicated in homogenization buffer A [20 mM Tris–HCl (pH 8.0), 10 mM EGTA, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 mM NaF and protease inhibitor cocktail] for 10 s on ice. The lysate was then centrifuged at 100 000 g for 1 h at 4°C. The supernatant was collected as the cytosolic fraction. The pellet was then resuspended in homogenization buffer B (with 1% Triton X-100) in buffer A) and sonicated for another 10 s on ice. The suspension was centrifuged at 15 000 g for 15 min at 4°C. The supernatant was collected as the membrane fraction. Using 8% SDS–polyacrylamide gel electrophoresis the protein was transferred to a PVDF membrane (Millipore, Bedford, MA) and subsequently hybridized with anti-PKCδ and anti-PKCζ antibodies. The blots were detected using the enhanced chemiluminescence method (Pierce). The blots were scanned using Kodak Image Station (New Haven, CT) and the densitometric measurements of the bands were performed using Kodak 1D 3.5 software.

PKC kinase assay

PKC kinase assay was performed according to a published method (28) with modifications. Briefly, cells were harvested 30 min after UVB irradiation in PKC lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Tween-20, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1 mM PMSF, 1 mM NaF, 0.1 mM Na3VO4, 10 mM β-glycerophosphate and protease inhibitor cocktail]. The cell lysate was then centrifuged at 12 000 g for 15 min at 4°C and the supernatant was collected as cellular protein. Four hundred micrograms of protein were immunoprecipitated with anti-PKC antibodies (4 µg) overnight and followed by incubation with protein G-sepharose for 1 h. The immunoprecipitates were washed five times with ice-cold kinase buffer 50 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM DTT, 2.5 mM EGTA, 1 mM NaF, 0.1 mM Na3VO4 and 10 mM β-glycerophosphate. The kinase assay was initiated by adding 30 µl of kinase buffer containing 10 µg of GST-MARCKS substrate, 0.5 µCi of [γ-32P]ATP and protease inhibitor cocktail. The reaction was performed for 30 min at 30°C and was terminated by adding 3 × sampler buffer. All reaction mixes were then boiled for 5 min before being separated on 10% SDS–polyacrylamide gel in Mini-Protein II system (Bio-Rad). Gels were then dried and exposed to an X-ray film (Kodak) at room temperature.

Statistical analysis

All numeric data are presented as mean ± standard deviations (SD) from at least three independent experiments and analyzed using one-way ANOVA with Student-Newman–Keul as post hoc comparison. A P-value < 0.05 is considered as statistically significant.

Results

PN sensitizes UVB-induced apoptosis via PKC-dependent pathways

Our previous findings demonstrated that PN is capable of sensitizing JB6 cells to UVB-induced apoptosis via the MAPK-AP-1 signaling pathway (12). It is known that UVB activates PKC, and PKC regulates the MAPK-AP-1 signaling pathway (29). Here we first investigated if PKC plays a role in PN-sensitized UVB-induced apoptosis by using 2 PKC inhibitors. As shown in Figure 1A, pre-treatment with GF109203X, a pan-PKC inhibitor also sensitizes cells to UVB-induced apoptosis, although to a lesser extent than PN. In contrast, pre-treatment with a specific PKCδ inhibitor, rotterlin, is capable of completely protecting cells from apoptosis induced by UVB alone or PN plus UVB (Figure 1B). Data from this study thus suggested that PKCδ is critical in both UVB and PN-UVB-induced apoptosis, consistent with some of the earlier reports that PKCδ possesses mainly pro-apoptotic functions in response to various extracellular stimuli (21,22).

PN selectively regulates different isoforms of PKC in UVB-induced activations

UVB is known to activate certain subtypes of PKC such as PKCδ and PKCζ (21,23). One of the critical events of PKC activation is the translocation from the cytosol to membrane (30). In this study, we first measured UVB-induced PKC activation by determining PKC membrane translocation. Figure 2 provides convincing evidence that UVB-induced membrane translocations of PKCδ and PKCζ 15 min post-irradiation in JB6 cells. Similar changes were also observed in other PKC isoforms such as PKCη and PKCα (data not shown). When cells were pre-treated with 5 µM of PN, the UVB-induced translocation of PKCζ was significantly inhibited (Figure 2B) while the translocation of PKCδ was further enhanced (Figure 2A). A consistent pattern of changes was also detected when the activation of PKC was measured using the in vitro PKC kinase assay; PN inhibited the UVB-induced activation of PKCζ (Figure 3B) while further enhancing that of PKCδ (Figure 3A).

In order to further understand the differential roles of PKC isoforms on the sensitization effect of PN on UVB-induced apoptosis, cells were transiently transfected with wild-type or DN forms of PKCδ or PKCζ plasmids, together with pDsRed.
as the transfection marker, followed by PN-UVB treatment. When the morphological changes of apoptotic cell death were examined under an inverted fluorescence microscope, it was found that the DN-PKCδ transfected cells became rather resistant to PN-UVB-induced apoptosis whereas the wild-type PKCδ transfected cells underwent massive apoptosis upon PN-UVB treatment (Figure 4). On the contrary, overexpression of wild-type PKCζ offered significant protection against PN and UVB-induced apoptosis (Figure 4). Such findings are basically consistent with the effect of PKC inhibitors as shown earlier (Figure 1A and B).

In order to obtain more quantitative data, we used another approach by analyzing DNA content/sub-G1 cells among those transfected cells (Figure 5A). Being consistent with the morphological changes, cells transfected with DN-PKCδ or wild-type PKCζ were resistant to PN and UVB-induced apoptotic cell death (Figure 5B and C). In contrast, overexpression of wild-type PKCδ or DN-PKCζ significantly enhanced cell death induced by PN and UVB treatment (Figure 5B and C).

Fig. 1. Involvement of PKC in cell death induced by PN-UVB. (A) Pre-treatment with a pan-specific PKC inhibitor (GF109203X); (B) pre-treatment with a specific PKCδ inhibitor (rotterlin). JB6 cells were pre-treated with 5 μM PN for 2 h, 20 μM GF109203X or 1 μM rotterlin for 1 h and then subjected to 50 mJ/cm² of UVB. In some groups, cells were first pre-treated with GF109203X or rotterlin for 1 h, followed by PN and UVB. Apoptosis was quantified with DNA content/sub-G1 analysis 24 h after UVB irradiation. Data were presented in mean ± SD from three independent experiments. An asterisk indicates statistically significant comparison with the untreated control group (P < 0.05).

Fig. 2. PKC activation in cells treated with PN and UVB measured by PKC membrane translocation. (A) PN enhanced UVB-induced PKCδ membrane translocation; (B) PN inhibited UVB-induced PKCζ membrane translocation. Cells were pre-treated with 5 μM PN for 2 h, 20 μM GF109203X or 1 μM rotterlin for 1 h and then subjected to 50 mJ/cm² of UVB. Cells were harvested 15 min after UVB irradiation. Using 8% SDS–polyacrylamide gels 30 μg of cytosolic or membrane proteins were separated and blotted with respective anti-PKC antibodies. The blots were scanned and the densitometric measurements of the bands were performed.

Fig. 3. PKC activation in cells treated with PN and UVB measured by PKC kinase assay. (A) PN enhanced UVB-induced PKCδ kinase activity; (B) PN inhibited UVB-induced PKCζ kinase activity. Cells were treated as described in Figure 2 and harvested 30 min after UVB irradiation. Cell lysate was immunoprecipitated with anti-PKCs antibodies and then subjected to PKC kinase assay as described in Materials and methods. Data were quantified as described in Figure 2.
PKCζ acts upstream of p38 MAPK but not JNK

Our previous findings illustrated that the MAPK-AP-1 pathway is one of the molecular targets of PN (12). Certain PKC isoforms have been shown to regulate the MAPK signaling cascade. For example, PKCδ has been reported to affect the UVB-induced phosphorylations of Erk 1/2 and JNK (21) while PKCζ primarily targets Erk 1/2 (25). Since PKCs are known to respond differently depending on cell type and stimuli, we set out to identify the effect of PKCδ and ζ on MAPK activation in our experimental system. Here we first confirmed our previous findings that PN pre-treatment is capable of blocking UVB-induced p38 activation, similar to the effect of a specific p38 inhibitor SB203580 (Figure 6A). Interestingly, pre-treatment with a pan-specific PKC inhibitor, GF109203X, inhibited the UVB-induced p38 activation to a certain extent, while the PKCδ specific inhibitor, rotterlin, has no effect on p38 activation (Figure 6A). Next we examined the relationship between PKC and p38 activation using genetic approaches. When cells were transfected with wild-type or DN-PKCδ, neither UVB-induced p38 activation nor the inhibitory effect of PN was affected as compared with the pcDNA-transfected control (Figure 6B). Intriguingly, overexpression of wild-type PKCζ abolished the inhibitory effect of PN on UVB-induced p38 activation.

**Fig. 4.** Impact of PKC overexpressions on apoptosis induced by PN-UVB. JB6 cells were transiently transfected with pcDNA, wild-type (wt) or DN forms of PKCδ or PKCζ and a transfection marker pDsRed as described in Materials and methods. Twenty-four hours after transfection, cells were pre-treated with 5 µM of PN for 2 h and then subjected to 50 mJ/cm² of UVB. The morphological changes of apoptotic cell death were examined under an inverted fluorescence microscope 24 h after UVB irradiation. Cells with successful transfection were marked in red.

**Fig. 5.** Effect of PKC expression on apoptosis induced by PN-UVB. (A) Apoptotic cell death was determined with DNA content/sub-G1 analysis coupled with flow cytometry after DAPI staining. JB6 cells were first transiently transfected with various PKC plasmids and pDsRed as a transfection marker, followed by being treated with PN and UVB. In a total of 20000 cells from each group analyzed using flow cytometry, only those transfected cells with expression of the red fluorescence protein were then selected for further analysis for percentage of sub-G1 cells. (B) Effect of wild-type (wt)- and DN-PKCδ expression on PN-UVB-induced apoptosis in JB6 cells. (C) Effect of wild-type (wt)- and DN-PKCζ expression on PN-UVB-induced apoptosis in JB6 cells. In both (B) and (C), JB6 cells were first transfected for 24 h, followed by treatment with PN and UVB. Cells were collected for DNA content analysis 24 h after UVB irradiation. Data were presented in mean ± SD from four independent experiments. An asterisk indicates statistically significant comparison with the group transfected with pcDNA only (P < 0.05).
p38 phosphorylation, while the overexpression of DN-PKCδ even completely blocked UVB-induced p38 phosphorylation. Therefore, these data clearly suggest that it is PKCζ, but not PKCδ, that is responsible for UVB-induced p38 activation.

Since PN is able to inhibit the phosphorylation of JNK induced by UVB as shown previously (12), here we also examined whether PKC has any functional role in UVB-induced JNK activation. As shown in Figure 7A, the two PKC inhibitors had no effect on UVB-induced JNK activation. Furthermore, overexpressions of the two DN forms of PKCδ and PKCζ had no effect on either UVB-induced JNK activation or the inhibitory effect of PN (Figure 7B). Unlike the previous findings (21,25), the total levels of MAPKs are not affected by the overexpressions of either the wild-type or DN forms of PKCδ and PKCζ. It is thus believed that UVB-induced JNK activation is independent of PKC activation.

In order to further confirm the functional linkage between PKCζ and p38 activation in protection of apoptosis induced by PN and UVB, JB6 cells were transiently transfected with the wild-type PKCζ together with DN-p38δ and DN-p38ζ2 plasmids. As shown in Figure 8A, overexpression of DN-p38ζ completely inhibited UVB-induced p38 activation. More importantly, while the overexpression of wild-type PKCζ offered significant protection against apoptosis induced by PN-UVB, the co-transfection of the DN forms of p38 protein abolished the protective effect of wild-type PKCζ and greatly sensitized cells to PN-UVB-induced apoptosis (Figure 8B). Such observations thus provide strong evidence that the anti-apoptotic function of PKCζ is achieved via p38 activation and PN is likely to act through PKCζ to suppress...
p38 activation, and then enhance apoptosis in UVB-treated JB6 cells.

**Discussion**

Even though the underlying mechanism(s) of the carcinogenic ability of UVB have not been fully understood, a number of molecular targets have been identified to be involved in UVB carcinogenesis, which include AP-1, NF-κB, NF-AT and STATs (16–19). We have previously shown that PN possesses a strong chemopreventive property against UVB-induced skin cancer via its potent inhibitory effect on the MAPK-AP-1 signaling pathway (12). Here we further demonstrated that PN selectively regulates the activities of PKC: promotion of the pro-apoptotic PKCδ and suppression of the pro-survival PKCζ. Moreover, we also provided evidence that PN acts on PKCζ upstream of p38 to sensitize UVB-induced apoptotic cell death. Thus, we reveal a new mechanism involved in the anticancer function of PN.

PKC can be classified into 11 isoforms based on the co-factors required for activation. UVB is known to activate certain PKC isoforms such as PKCδ, ε, ζ, λ/ and η, leading to apoptosis or cell survival (21,23,25,31). It has been reported that UVB induces phospholipase A2 activation and arachidonic acid release, and activates PKC (32). Furthermore, UVB irradiation of cultured cells is also known to elevate levels of DAG (33) as well as to generate reactive oxygen radicals that may activate PKC (34). Among all the UVB-activated PKC isoforms, PKCδ seems to be the main subtype involved in apoptotic signaling induced by various stimuli including UVB (22). It is generally believed that activated PKCδ decreases mitochondrial membrane potential, resulting in cytochrome c release, caspase activation and apoptosis (22). On the other hand, PKCζ has been shown to promote cell survival by either stimulating the nucleotide excision repair activity (35) or phosphorylating Rel A and subsequently activating the NF-κB survival pathway (36).

One important observation of this study is the differential effect by PN on different PKC isoforms in cells treated with UVB: PN selectively enhances the pro-apoptotic PKCδ and suppresses the anti-apoptotic PKCζ (Figures 2 and 3). Indeed, selective effects on different PKC isoforms have been reported previously. Sodium butyrate has been shown to upregulate PKCζ while downregulating PKCδ during erythroid differentiation (37). UCN-01, a staurosporine analog, has been illustrated to have selective effect on different PKC isoforms used in vitro kinase assay (38). In addition, a diazene carbol derivative diamide, which oxidizes thiols to disulfides through addition/displacement reactions at the diazene bond, stimulates the pro-apoptotic PKCδ while inactivating the oncogenic PKCζ (39).

The selective regulation on PKCδ and PKCζ can also be appreciated by the difference in their molecular structures. Although both PKCζs contain an N-terminal regulatory domain and a C-terminal catalytic kinase domain, PKCδ contains two cysteine-rich C1 subdomains (40) whereas PKCζ has only one zinc finger binding region (41). DAG is known to bind to one of the cysteine-rich C1 subdomains and activate PKCδ. In contrast, PKCζ is regulated by other lipid co-factors such as phosphatidylinositol (PI) 3,4,5-P_3 and ceramide (42). The exact mechanisms responsible for the differential regulation of PN on UVB-induced PKC activation are currently not known. One possible explanation is related to the involvement of the caspase cascade in PKC activation. For instance, PKCδ is cleaved by caspase-3 during apoptosis to a more catalytically active fragment (43). In contrast, caspase-3-dependent cleavage of PKCζ generates a fragment that corresponds to its catalytic domain and is enzymatically inactive (24). Since PN is a potent activator of caspase-3 (8,9), it is possible that
Both PKCδ and PKCζ were cleaved by caspase-3 in response to PN-UVB treatment. As a result, the catalytically active PKCδ and the inactive fragment of PKCζ are generated, leading to more profound cell death induced by PN-UVB.

Currently, there is still controversy with regard to the exact role of JNK and p38 in UVB-induced apoptosis, with both pro- and anti-apoptotic activities being reported (44-46). Our previous findings indicated that PN sensitizes UVB-induced apoptosis by inhibiting both JNK and p38, leading to blockade in the pro-survival AP-1 pathway (12). The link between MAPK and PKC has been reported previously. Chen et al. (21) showed that PKCδ and PKCe mediate UVB-induced signal transduction and apoptosis through the activation of ERK and JNK. Furthermore, inhibition of PKCα/δ with a dominant negative mutant suppressed UVB-induced ERK and the subsequent AP-1 activation (47). On the other hand, the antisense oligonucleotide of PKCζ has been shown to inhibit UVB-induced AP-1 activation (25). In this study, we provide convincing evidence suggesting that PKCζ acts upstream of p38, but not JNK, to protect cell death induced by PN-UVB, based on experimental data using both pharmacological and genetic approaches. For instance, overexpression of wide-type PKCζ reverses the inhibitory effect of PN on UVB-induced p38 phosphorylation (Figure 6C), and transfections with wild-type PKCζ together with DN-p38α and DN-p38β2 plasmids completely abolishing the protective effect of wild-type PKCζ and greatly sensitizes cells to PN-UVB-induced apoptosis (Figure 8B). Therefore, it is clear that the sensitization activity of PN on UVB-induced apoptosis is probably achieved through its inhibitory effect on the PKCζ and p38 signaling pathway.

In summary, we show for the first time that PN sensitizes JB6 cells to UVB-induced apoptosis through selective regulation on the pro-apoptotic PKCζ and the pro-survival PKCζ functions. Furthermore, our data also suggest that the UVB-induced p38 MAPK activation is regulated via a PKCζ-dependent mechanism. These findings may shed new light in understanding the anticancer activity of PN.

Acknowledgements

The authors thank Dr J.W. Soh for providing PKC plasmids, Dr J.Han for giving the DN-p38α and DN-p38β2 plasmids, Drs W.Duan and Y.M.Zhu for their help on PKC kinase assay. We also thank S.Y. Zhang, Q.Huang, R.X. Shi, M.Zhao and Y.B.Ong for their technical assistance. Y.K.W. is supported by a research scholarship from the National University of Singapore. This work is supported by a research grant from NUS Academic Research Fund.

Conflict of Interest Statement: None declared.

References

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Received March 17, 2005; revised July 10, 2005; accepted July 22, 2005