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A polypeptide from *Chlamys farreri* inhibits UVB-induced HaCaT cells apoptosis via the Apaf-1/caspase-9 and Smac/XIAP signaling pathway*

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Abstract A novel marine active polypeptide (PCF), isolated from the gonochoric Chinese scallop, *Chlamys farreri*, has potential antioxidant and anti-apoptotic activity against ultraviolet irradiation. We investigated whether UVB-induced HaCaT cell apoptosis occurs via the mitochondrial pathways Apaf-1/caspase-9 and Smac/XIAP/caspase-3. We then investigated the molecular mechanisms controlling the anti-apoptotic effect of PCF. Pre-treatment with PCF and caspase-9 inhibitor significantly inhibited UVB-induced apoptosis in HaCaT cells based on a DNA fragmentation assay and Hoechst 33258 staining. The expression of Apaf-1 and the cleavage of procaspase-9 were dose-dependently reduced by 1.42–5.96 mmol/L PCF pretreatment in UVB-irradiated HaCaT cells. This was followed by inhibition of cleavage of procaspase-3, whose activation induced cell apoptosis. Meanwhile, PCF significantly and dose-dependently enhanced the activation of ATPase. Furthermore, we demonstrated that PCF strongly inhibited the release of Smac from the mitochondria to cytosol by reducing the degradation of XIAP dose-dependently. We conclude that the protective effect of PCF against UVB irradiation in HaCaT cells may be attributed to the inhibition of the Apaf-1/caspase-9 and Smac/XIAP/caspase-3 apoptotic signaling pathways.

Keyword: polypeptide from *Chlamys farreri* (PCF); UVB; apoptosis; Smac/XIAP; Apaf-1/caspase-9

1 INTRODUCTION

Several studies have shown that UV radiation, in particular UVB (290–320 nm) directly induces genetic alteration, which in turn may promote neoplastic transformation of the skin and apoptosis (Murphy et al., 2001). The induction of apoptosis in keratinocytes by ultraviolet irradiation is considered to have a protective function against skin cancer formation. UV-induced DNA damage is a crucial event in UVB- and UVC-mediated apoptosis (Takasawa et al., 2005). Apoptosis can be triggered by diverse intrinsic and extrinsic signals. Initiation of apoptosis typically follows a cascade of signaling events or apoptotic pathways upon receipt of a variety of apoptotic signals. Apoptogenic stimuli, including UV irradiation and cytokines, are mediated by the release of cytochrome c from mitochondria into the cytosol (Adrain et al., 2001) and its subsequent interaction with apoptotic protease-activating factor-1 (Apaf-1). The cytochrome c/Apaf-1 complex in turn activates the initiator caspase-9 and, subsequently, the effector caspase-3, leading to apoptosis. This last step is dependent on the availability of dATP/ATP (Kim et al., 2008). However, questions have been raised regarding the necessary concentrations of ATP and cytochrome c during apoptosome formation, as well as caspase-9 activation. Studies that have focused on UV

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irradiation and cells in this apoptotic pathway have concentrated primarily on UVC radiation and tumor cells. Little is known about UVB-induced cell apoptosis via the cytochrome c/Apaf-1/caspase-9/ caspase-3 mitochondrial pathway, particularly in HaCaT keratinocytes.

Recent studies have shown that the inhibitors of apoptosis proteins (IAPs) suppress apoptosis by binding directly to caspase molecules and inhibiting their activity (Deveraux et al., 1999). Several members of the human IAP family of proteins, including X chromosome-linked IAP (XIAP), cellular IAP (c-IAP1), and c-IAP2, are potent direct inhibitors of Caspase-3, -7, and -9. Among these, XIAP is the most potent inhibitor of caspases and apoptosis (Suzuki et al., 2004). Furthermore, Smac/DIABLO is another important regulator of apoptosis. The sustained release of this protein from the mitochondria into the cytosol, upstream of caspase activation, plays a determinant role in the onset of apoptosis induced by high dose UVB irradiation (Takasawa et al., 2003). The molecular mechanisms by which HaCaT cells, exposed to low dose UVB irradiation, are irreversibly committed to the apoptotic process have not yet been identified.

A polypeptide from *Chlamys farreri* (PCF, Mr = 879 kDa) is a novel marine active material isolated from the gonochoric Chinese scallop *C. farreri* (Appl: 00111426.9, China). Recent studies in our laboratory suggest that PCF inhibits the production of ROS, leading to inhibition of cytochrome c release and apoptosis of HaCaT cells after UVB exposure (Li et al., 2007). In the current study, we used the apoptotic model of UVB-induced HaCaT cells to investigate whether UVB-induced HaCaT cell apoptosis is mediated by the mitochondrial pathways Apaf-1/ caspase-9 and Smac/XIAP/ caspase-3. We also investigated mechanism controlling the anti-apoptotic effect of PCF.

2 MATERIALS AND METHODS

2.1 Materials

PCF was purified (purity >96%) and analyzed by HPLC (Yellow Sea Fishery Research Institute, China), dissolved in sterile deionized water, and stored at 4°C. Anti-Smac antibody was obtained from eBioscience (Beverly, MA, USA). Anti-β-actin, caspase-9 and Apaf-1 antibodies were purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China). Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse) were purchased from Wuhan Boster Biological Technology Co. Ltd. (Wuhan, China) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. The primers for XIAP and GAPDH were designed and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, China. All the other chemicals were of the highest grade commercially available.

2.2 Cell culture and UVB treatment

The human keratinocyte cell line, HaCaT, was grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were randomly divided into several groups including a control group (normal cultured cells), a model group (UVB-irradiated cells), a PCF group (PCF pretreated and then UVB-irradiated cells) and an inhibitor group (the corresponding inhibitor pretreated and then UVB-irradiated cells). When the cells were reached 80%–90% confluence, they were exposed to UVB for 0.5 h (20 mJ/cm^2) with UVB lamps (Beijing Normal University, China). We added PCF to the medium 2 h before irradiation. During irradiation, the medium was discarded and the cells were placed in PBS. After irradiation, the medium was changed to DMEM and the cells were cultured normally.

2.3 Hoechst 33258 staining

Eighteen hours after UVB treatment, the cells were collected and fixed, washed twice with PBS, and stained with Hoechst 33258 staining solution, based on the manufacturer's instructions (Hoechst Staining Kit, Beyotime Biotechnology, China). The apoptotic features of cell death were determined by the staining of cell nuclei with the DNA-binding fluorochrome Hoechst 33258 and assessment of chromatin condensation using a fluorescence microscope (Leica DBI 4000 B). In each group, six microscopic fields were selected randomly and five hundreds cells were counted. Apoptotic cell death was then calculated as a percentage of apoptotic cells over the total blue fluorescent protein-positive cells.

2.4 DNA fragmentation assay

We performed a DNA fragmentation assay based on previous descriptions. Briefly, cells were washed with PBS and harvested 18 h after UVB irradiation. The cell pellets were then incubated in cell lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5, 10 mmol/L EDTA, 0.5% SDS, and 500 mg/L

Proteinase K) overnight at 50°C. After incubation, the cell lysate was extracted with phenol/chloroform/ isopropyl alcohol (25:24:1, v/v). The DNA was precipitated with ethanol in the presence of sodium acetate at -20°C overnight, and then washed with 70% ethanol. The DNA pellets were dissolved in TE buffer and incubated with RNase A (20 μg/L) at 37°C for 30 min. DNA fragmentation was analyzed by electrophoresis on 1.5% agarose gels, staining with ethidium bromide, and visualization under UV light.

2.5 Total and Cytosolic protein extraction

After UVB treatment, the HaCaT cells were harvested, total cellular proteins were extracted with in ice-cold lysis buffer (20 mmol/L Tris-HC1, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% TritonX-100, 2.5 mmol/L sodiumpyropho sphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 mg/L leupeptin, 1 mmol/L PMSF). The mixture was centrifuged at 10 000 g for 10 min at 4°C. The supernatant was collected to represent the total protein. The UVB treated cells (1×10^6) were pelleted and resuspended in 300 μL of buffer (Mannitol 220 mmol/L, Sucrose 68 mmol/L, Sodium pyruvate 5 mmol/L, HEPES-NaOH (pH 7.2) 10 mmol/L, NaCl 2 mmol/L, EGTA 0.5 mmol/L, KH_2PO_4 2.5 mmol/L, $MgCl_2$ 2 mmol/L, PMSF 0.1 mmol/L, DTT 1 mmol/L) for cytosolic protein extraction. The cells were then vortexed 10–15 times. After centrifugation (750 g for 3 min), the supernatant was collected and re-centrifuged (10 000 g for 5 min). The resulting supernatant represented the cytosolic extract. The protein concentration was measured using a BCA protein assay kit (Beyotime Biotechnology, China).

2.6 Western blot analysis for Smac, Caspase 9 and Apaf-1

Smac, Caspase 9 and Apaf-1 were analyzed by Western blotting. After UVB treatment, the protein was extracted and quantified as described previously. An equal amount of protein was separated on a 10% SDS-PAGE and transferred electrophoretically to the nitrocellulose membranes. The membrane was blocked with 5% bovine serum albumin and 0.1% Tween-20 in Tris-buffered saline for 2 h at room temperature. The blots were incubated with antibodies against Smac, Caspase 9, Apaf-1, or β-actin (dilution 1:200) overnight at 4°C, and then with peroxidase-conjugated secondary antibodies (dilution 1:500) for 40 min at room temperature. The bands were detected using the diaminobenzidine detection kit (Boster Biotechnology, Wuhan, China). The results were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 Detection of XIAP by RT-PCR

Total RNA was extracted from HaCaT cells according to the manufacturer's protocol with Trizol reagent (Takara, China). The specific oligonucleotide primer sequences were as follows: TTC CGG CCC AAA ACA AAG A and ATA TAC CCG AGG AAC CCT GCC for human XIAP (Sangon Biological Engineering Technology, Shanghai, China); CGT GGA AGG ACT CAT GAC CA and TCC AGG GGT CTT ACT CCT TG for GAPDH (Sangon Biological Engineering Technology, Shanghai, China). DNA was amplified immediately with a single cycle at 95°C for 3 min and 35 cycles at 94°C for 40 s, 60°C for 40 s, and 72°C for 40 s, followed by a final extension at 72°C for 3 min. The PCR products were subject to a 1% agarose gel and photographed. The intensity of each RT-PCR band was analyzed by Quantity One software.

2.8 Detection of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase

The cells in the 6-well culture dishes were washed 3 times with ice-cold D-Hanks and lysed in the extraction buffer (50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L ethyleneglycol–*bis* (2-aminoethylether) tetraacetic acid, 150 mmol/L NaCl, 1% (*v/v*) Triton X-100, 1 mmol/L phenyl-methylsulfonyl, 10 μg/mL aprotinin, 10 mmol/L EDTA, 1 mmol/L NaF, and 1 mmol/L Na_3VO_4) on ice for 30 min. The cells were then scraped from the plates and the lysate was centrifuged at 20 000 g for 10 min at 4°C. The amount of protein in the cleared lysate was quantified by the bicinchoninic acid assay (Beyotime Biotechnology, China). After determining the amount of total protein in the supernatant, we detected Na^+-K^+-ATPase and Na^+-Ca^{2+}-ATPase using biochemical kits, following the manufacturer's instructions (Nanjing Institute of Jiancheng Biological Engineering, Nanjing, China).

2.9 Statistical analysis

Data are expressed as means±SD. Differences between groups were analyzed by one-way ANONA, followed by the Bonferroni test using Origin 7.5 (Origin Lab Corporation, Northampton, MA, USA). The difference was considered significant if $P<0.05$.

3 RESULTS

3.1 Inhibitory effects of PCF and Z-LEHD-FMK on HaCaT cell apoptosis induced by UVB irradiation

To determine whether UVB-induced HaCaT cell

death was due to apoptosis, we examined the morphologic changes in cell nuclei and DNA fragmentation. Previously, we investigated the effect of UVB on HaCaT cells (Li et al., 2007). Here we noted marked morphologic changes in cell nuclei in cells that were incubated for 18 h after UVB irradiation (20 mJ/cm^2, 30 min) (Fig.1b) compared with the untreated controls (Fig.1a). Hoechst 33258-stained HaCaT cells had condensed nuclei, which is characteristic of apoptosis. The cleavage of chromosomal DNA into fragments is a biochemical hallmark of apoptosis. Thus, we examined DNA fragmentation using the DNA laddering assay. The induction of apoptosis in UVB-irradiated HaCaT cells was confirmed by the appearance of a DNA ladder (Fig.1, lane 1) 18 h after UVB irradiation at a dose of 20 mJ/cm^2. Pretreatment with 5.69, 2.84, or 1.42 mmol/L PCF for 2 h prior to irradiation markedly attenuated UVB-induced apoptosis (Fig.1c and lanes 4–6). The specific irreversible caspase-9 peptide inhibitor Z-LEHD-FMK also demonstrated anti-apoptotic activity (Fig.1d and lane 2), suggesting that caspase-9 participates in UVB-induced HaCaT cells apoptosis.

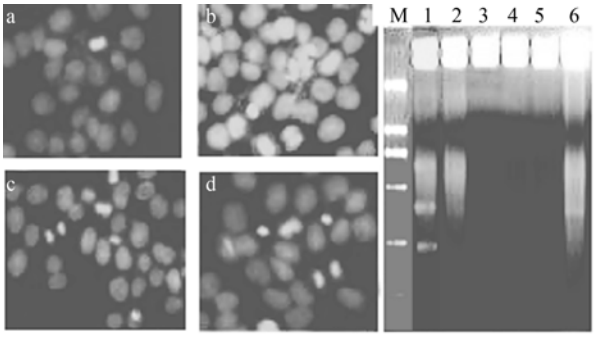

Fig.1 Effects of PCF and Caspase-9 inhibitor Z-LEHD-FMK on UVB-induced HaCaT cells apoptosis by Hoechst 33258 staining and DNA-fragment

a. control; b. UVB model; c. UVB+ Z-LEHD-FMK; d. UVB+PCF2 (2.84 mmol/L); M. marker; 1. UVB model; 2. UVB+ Z-LEHD-FMK 3. control; 4. UVB+PCF5.69 mmol/L; 5. UVB+PCF2.84 mmol/L; 6.UVB+ PCF1.42 mmol/L

3.2 PCF inhibited the cleavage of caspase-9 in UVB-induced HaCaT cells

Caspase-9 and -3 play critical roles in the mitochondrial apoptotic signaling pathway resulting from DNA damage (Sitailo et al., 2002). In our previous study, we reported that UVB radiation activates caspase-3 (Wang et al., 2004). Therefore, in the current study we assessed whether UVB radiation increased activation of procaspase-9 to caspase-9 at 14 h in the cytosolic compartment of HaCaT cells. UVB radiation caused the cleavage of caspase-9 (Fig.2). Pre-treatment with 1.42–5.69 mmol/L PCF significantly inhibited the cleavage of caspase-9 in a dose-dependent manner.

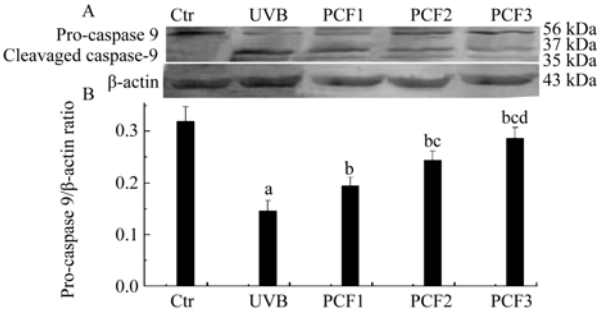

Fig.2 Effect of PCF on UVB-induced Caspase-9 protein expression in HaCaT cells

A, Ctr-control; UVB, Cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3, Cells were pretreated with 1.42, 2.84, or 5.69 mmol/L PCF for 2 h prior to UVB irradiation. After irradiation, the cells were incubated for 14 h and Caspase-9 was detected by western blot. Results are representative of three independent experiments. (B) Quantification of (A) results. Results are expressed as the ratio of expression level of Caspase-9 over β-actin. $^aP<0.01$, vs. control; $^bP<0.01$, vs. UVB; $^cP<0.05$ vs. UVB+PCF1; $^dP<0.05$, vs. UVB+PCF2

3.3 PCF reduced the expression of Apaf-1 in UVB-induced HaCaT cells

Previous studies using cell-free systems have demonstrated that cytochrome c is a regulatory protein that binds to the CED-4 like adaptor protein Apaf-1, generating a high molecular weight protein complex termed apoptosome. Apoptosome then activates the initiators CASP-9, -3, and -7 (Li et al., 1997). We demonstrated that PCF inhibits the UVB induced release of cytochrome c from mitochondria into the cytosol in HaCaT cells. Therefore, we evaluated whether UVB radiation actives Apaf-1 induced oligomerization of Apaf-1 and then recognizes inactive procaspase-9, forming the apoptosome and triggering autocatalytic processing of procaspase-9. UVB irradiation increased levels of Apaf-1 14 h post-irradiation compared with the control group (Fig.3). Pretreatment with 1.42–5.69 mmol/L PCF significantly decreased the expression of Apaf-1 dose-dependently.

3.4 PCF increased the activity of ATPase in UVB-induced HaCaT cells

It is generally accepted that ATP and cytochrome c activate caspase-9 together. Recent studies also indicate that ATP-dependent regulation of caspase-9 activation may represent an additional level of control down-stream from cytochrome c (Riedl, 2007). A recent study demonstrated that the Na^+-K^+-ATPase (Na^+ K^+-pump) plays a critical role in maintaining

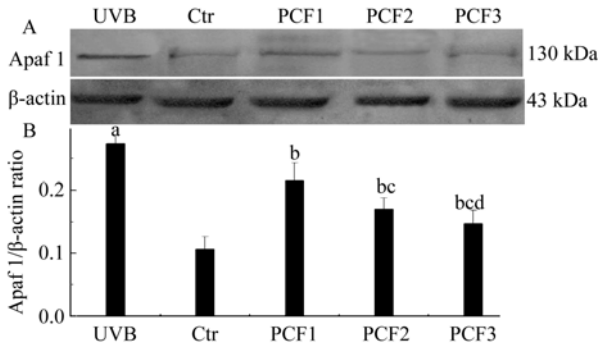

Fig.3 Effect of PCF on UVB-induced Apaf-1 protein expression in HaCaT cells

A, Ctr-control; UVB-Cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3, Cells were pretreated with 1.42, 2.84, or 5.69 mmol/L PCF for 2 h prior to UVB irradiation. After irradiation, the cells were incubated for 14 h and Apaf-1 was detected by western blot. Results are representative of three independent experiments. (B) Quantification of (A) results. Results are expressed as the ratio of expression level of Apaf-1 over β-actin. [a]P<0.01, vs. control; [b]P<0.01, vs. UVB; [c]P<0.05 vs. UVB+PCF1; [d]P<0.05, vs. UVB+PCF2

ion homeostasis. Blocking the Na^+ K^+-pump may lead to neuronal cell apoptosis due to oxidant stress (Wang et al., 2003). To evaluate the role of ATP in apoptosis, we measured Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activity. In UVB-damaged cells, the activity of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase decreased significantly (Table 1). Pretreatment with 1.42–5.69 mmol/L PCF increased the levels of ATPase dose-dependently.

Table 1 Effect of PCF on UVB-induced activities of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase in HaCaT cells ($\bar{x} \pm s$, n=3)

Groups	Na^+-k^+-ATPase activity (%)	$Ca2^+$-$Mg2^+$-ATPase activity (%)
Ctr	171.48±3.99	58.32±4.07
UVB	121.37±4.14[a]	25.46±4.38[a]
UVB+PCF1	134.88±4.28[b]	34.72±3.04[b]
UVB+PCF2	152.17±6.52[bc]	42.05±2.70[bc]
UVB+PCF3	160.64±2.51[bcd]	50.50±2.78[bcd]

[a]P<0.01, vs. control; [b]P<0.01, vs. UVB; [c]P<0.05 vs. UVB+PCF1; [d]P<0.05, vs. UVB+PCF2

3.5 PCF suppressed the degradation of XIAP in UVB-induced HaCaT cells

Recent studies have shown that IAPs, particularly XIAP, suppress apoptosis by binding directly to caspase molecules and thus inhibiting caspase activity (Deveraux et al., 1999). The degradation of XIAP eventually leads to a diminishing of its caspase inhibitory activity, resulting in an apoptotic response. Given this, we examined the changes in mRNA XIAP levels using RT-PCR. We observed a significant decrease in mRNA XIAP levels in UVB-irradiated cells 12 h after the treatment (20 mJ/cm^2) (Fig. 4). Pretreatment with 1.42–5.69 mmol/L PCF effectively inhibited the degradation of XIAP dose-dependently.

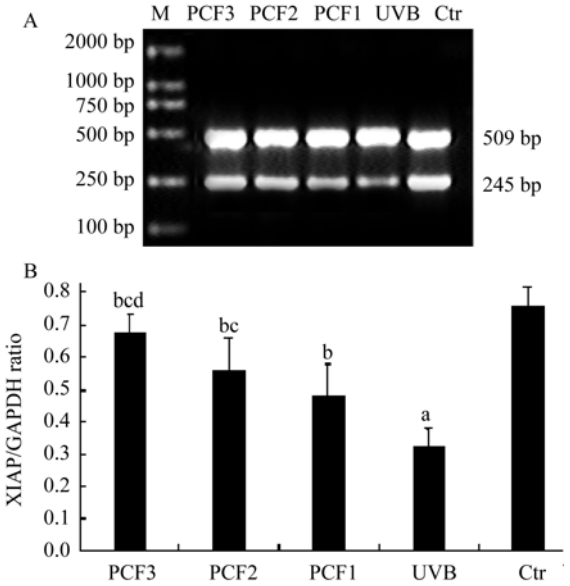

Fig.4 Effect of PCF on UVB-induced XIAP mRNA expression in HaCaT cells

A, Ctr-control; UVB-Cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3, Cells were pretreated with 1.42, 2.84, or 5.69 mmol/L PCF for 2 h prior to UVB irradiation. After irradiation, the cells were incubated for 12 h and XIAP was detected by RT-PCR. Results are representative of three independent experiments. (B) Quantification of (A) results. Results are expressed as the ratio of expression level of XIAP over GAPDH. [a]P<0.01, vs. control; [b]P<0.01, vs. UVB; [c]P<0.05 vs. UVB+PCF1; [d]P<0.05, vs. UVB+PCF2

3.6 PCF inhibited the release of Smac from mitochondria into the cytosol in UVB-induced HaCaT cells

Smac/DIABLO, is another important regulator of apoptosis induced by UVB irradiation. Smac/DIABLO is released from the mitochondria into the cytosol during apoptosis (Chai et al., 2000). It functions by neutralizing the inhibitory effects of IAPs on caspases (Du et al., 2000). Therefore, we measured the UVB irradiation-triggered release of Smac/DIABLO from the mitochondria into the cytosol in HaCaT cells. Equal aliquots of the cytosolic fractions used for the examination of Smac release were subjected to Western blot analysis using anti-Smac/DIABLO antibody. UVB irradiation resulted in a significant increase in the Smac/DIABLO level in the cytosol 8 h after irradiation (Fig.5). Pretreatment with 1.42–5.69 mmol/L PCF dose-dependently inhibited the increase in Smac/

DIABLO levels in the cytosol. Our results suggest that PCF suppresses the release of Smac/DIABLO from the mitochondria into the cytosol in UVB-exposed HaCaT cells.

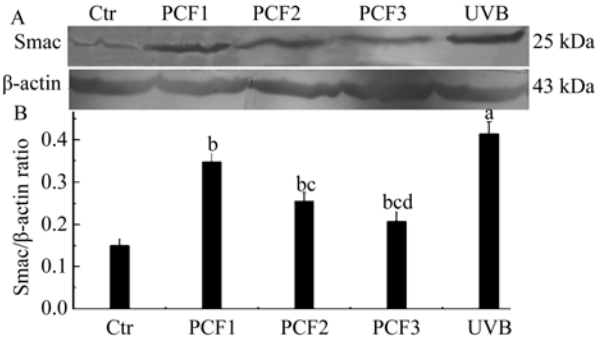

Fig.5 Effect of PCF on UVB-induced Smac protein expression in HaCaT cells

A, Ctr-control; UVB-Cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3, Cells were pretreated with 1.42, 2.84, 5.69 mmol/L PCF for 2 h prior to UVB irradiation. After irradiation, the cells were incubated for 8 h and Apaf-1 was detected by western blot. Results are representative of three independent experiments. (B) Quantification of (A) results. Results are expressed as the ratio of expression level of Smac over β-actin. $^aP<0.01$, vs. control; $^bP<0.01$, vs. UVB; $^cP<0.05$ vs. UVB+PCF1; $^dP<0.05$, vs. UVB+PCF2

4 DISCUSSION

Apoptosis is a neat, orderly process characterized by overall shrinkage in the volume of the cell and its nucleus, the loss of adhesion to neighboring cells, cell membrane blebbing, DNA fragmentation, changes in mitochondrial structure, and phosphatidylserine externalization (Steller, 1995). Based on our observations of morphologic changes in the cellular nuclei and DNA fragmentation, UVB irradiation induced apoptosis in the HaCaT cells. This indicates that we successfully imitated the UVB irradiation induced apoptosis model of HaCaT cells. PCF effectively reduced UVB-induced apoptosis in the HaCaT cells *in vitro* in a dose-dependent manner. Consistent with our previous research, both the DNA fragmentation assay and Hoechst 33258 demonstrated that PCF is an anti-apoptotic reagent for HaCaT cells against UVB irradiation.

Caspase family proteases play an essential role in the process of apoptosis. Procaspase-9 is recruited and activated by the mitochondrial pathway (Bratton et al., 2001). Procaspase-9 may form a complex with cytochrome c and apoptotic protease activating factor-1 (Apaf-1) in the cytosol in the presence of dATP or ATP, and it is the critical player of the apoptotic stimuli acting through mitochondrial dysfunction (Shimizu et al., 1999). The complex then activates caspase-9 which is proteolytically cleaved by procaspase-9, an “initiator caspase” that in turn activates the “effector caspases” (caspase-3, -6 and -7). This process culminates in apoptotic cell death. We found that UVB irradiation caused the cleavage of procaspase-9 to caspase-9 14 h after irradiation. The activity of caspase-9 was suppressed by Z-LEHD-FMK, causing inhibition of UVB-induced apoptosis. Previously we demonstrated that UVB radiation activates caspase-3 after 18 h (Wang et al., 2004), suggesting upstream regulation of caspase-3 activation. Previous studies have suggested that Apaf-1, which can simultaneously bind to procaspase-9, is required for mitochondria-dependent apoptosis (Soengas et al., 1999). We noted that UVB radiation up-regulated the expression of Apaf-1 14 h post-irradiation in HaCaT cells, suggesting downstream regulation of cytochrome c as cytochrome c was activated at 6 h. Therefore, we inferred that the mitochondrial pathway cytochrome c/Apaf-1/caspase-9/ caspase-3 was involved in UVB induced HaCaT cells apoptosis.

ATP is a key molecule for chromatin condensation, nuclear fragmentation and regulation, and maintenance of ion homeostasis during apoptosis. UVB radiation significantly decreased the activity of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase in HaCaT cells. This, in turn, causes a decrease in intracellular K^+ concentration and an increase in intracellular Ca^{2+} levels, a common feature of apoptosis. Changes in the level of both cytosolic cations (calcium and potassium) and alterations in the level of ATP regulate the apoptotic process by controlling the mitochondrial membrane potential and activity of caspase-like proteases and endonucleases. This is consistent with the findings of Nilkantha Sen et al. who reported that Camptothecin-induced oxidative stress also causes impairment of the Na^+-K^+-ATPase and Ca^{2+}-ATPase, and subsequently increases apoptosis levels in leishmanial cells (Nilkantha et al., 2004).

Even in the presence of functional Apaf-1 and procaspase-9, inhibitors of apoptosis, such as XIAP (inhibitors of apoptosis proteins), can prevent the proteolytic processing of procaspase-3 by blocking the cytochrome c-induced activation of procaspase-9 (Du et al., 2000). Among IAPs, XIAP is the most potent and is characterized by the presence of three domains known as baculoviral IAP repeat (BIR) domains that are essential for inhibiting caspase activity (Deveraux et al., 1998). IAPs, in turn, are regulated by the mitochondrial protein Smac, which promotes apoptosis by removing XIAP from processed caspase-9 (Ekert et al., 2001). Our results

showed that a low dose of UVB radiation decreases XIAP levels 12 h post-irradiation and increases the expression of Smac in 8 h post-irradiation. The release of Smac from the mitochondria inhibited the function of XIAP, suggesting that Smac/DIABLO acts at during post-mitochondrial activation of caspases by enhancing the formation or activation of the apoptosome.

Taken together, our observations suggest that UVB irradiation damaged the activity of ATPases and induced the release of cytochrome c and Smac from the mitochondria into the cytosol, and then activated Apaf-1 and caspase-9. Meanwhile, the expression of XIAP was inhibited, thus activating caspase-3 and leading to apoptosis. PCF is known to prevent UV-induced HaCaT cells apoptosis via several cell signaling pathways, based on studies in our laboratory. Here, we evaluated the molecular mechanisms underlying the previously reported chemo-preventative effects of PCF on UVB-induced HaCaT cell apoptosis. Pretreatment with PCF in UVB-exposed HaCaT cells had multiple effects. PCF dose-dependently increased ATPase activity, decreased Apaf-1 expression, and inhibited the cleavage of caspase-9. In addition, PCF dose-dependently inhibited the release of Smac while suppressing the degradation of XIAP.

In conclusion, the mitochondrial pathways cytochrome c/Apaf-1/caspase-9 and Smac/XIAP/ caspase-3 are involved in UVB-induced HaCaT cell apoptosis. PCF exerted its anti-apoptotic effect by influencing the expression of signal molecules in this pathway. Our present observations provide new insight into the protective functions of PCF involved in the process of apoptosis in HaCaT cells induced by UVB.

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