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Effect of polypeptide from *Chlamys farreri* on UVB-induced ROS/NF-κB/COX-2 activation and apoptosis in HaCaT cells

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ABSTRACT

Polypeptide from *Chlamys farreri* (PCF) is a novel marine polypeptide compound isolated from gonochoric Chinese scallop *Chlamys farreri*, this study we further investigate the mechanisms of PCF exerting its anti-apoptotic effect. The results indicated that PCF, ROS scavenger NAC and NF-κB inhibitor MG132 effectively inhibited UVB-induced HaCaT cells apoptosis. PCF (2.84 mM) showed potential ROS scavenging activities in a kinetic process. PCF (1.42–5.69 mM) dose-dependently increased the expressions of Cu, Zn-SOD, CAT and GPx meanwhile decreased the expressions of p-NF-κB/p65 and COX-2 in UVB-induced HaCaT cells. Additionally, pretreatment with NAC significantly declined the generation of ROS and the expression of p-NF-κB/p65. We concluded that ROS, NF-κB and COX-2 are involved in UVB-induced HaCaT cells apoptosis, PCF exerts its protective effects via scavenging ROS, increasing the expression of antioxidative enzymes and inhibition the activation of NF-κB and COX-2.

1. Introduction

Excessive exposure of solar ultraviolet radiation, particularly its UVB component, to humans causes many adverse effects. UVB-induced free radical formation and subsequent lipid peroxidation are considered to be a major mechanism of UV irradiation-induced cutaneous photodamage [1]. UV irradiation is a potent inducer of various reactive oxygen species (ROS), including hydroxyl radicals ($OH^{\cdot}$), superoxide radicals O_2^-, and peroxyl radical and their active precursors namely singlet oxygen $^1O_2$, hydrogen peroxide (H_2O_2) and ozone [2], which play a role in the modulation of apoptosis [3]. Indeed, several studies have been shown UVB-induced ROS formation leading to apoptosis [4], but the dose of UVB irradiation was large and the measurement of intracellular ROS mainly using immunofluorescence, which could not be quantitated exactly; additionally, seldom report was performed about the kinetic analysis of intracellular ROS formation.

ROS have both intrinsic and extrinsic causes, and cells are protected by multiple levels of antioxidant defenses. In skin, they are constantly generated in keratinocytes and fibroblasts, and are rapidly removed by nonenzymic (ascorbic acid, tocopherol) and enzymic antioxidants (superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), thiredoxin reductase, and glutathione reductase) that maintain the pro-oxidant/antioxidant balance, thus resulting in cell and tissue stabilization [5,6]. However, overflow of ROS, extensively formed by the reaction of UV photons with endogenous photosensitizers in the skin, may overwhelm the antioxidant defence mechanisms resulting in pro-oxidant/antioxidant disequilibrium leading to deleterious effects on cells and therefore, contributing to UVB-induced apoptosis [7,8]. So antioxidant defense mechanisms against cell death involving apoptosis due to UVB irradiation in HaCaT cell lines should be studied.

Sung et al. demonstrated that UVB-induced ROS production in HaCaT cells, playing an essential role in the activation of NF-κB [9], which can induce a variety of cellular responses, including the induction of inflammation, cell proliferation, differentiation, or apoptosis, these divergent cell responses to NF-κB activation are defined by cell-specific and stimuli-specific mechanisms [10]. In most cases, NF-κB functions as a survival factor. But in HaCaT cells, because of the aberrant NF-κB activity increased the sensitivity of HaCaT cells to UVB-induced apoptosis [11]. It has been suggested that NF-κB plays an important role in cellular death after UV irradiation, so particular focus was directed at the mechanism to activate this transcription factor. NF-κB is a known positive regulator of COX-2 transcription, UVB significantly increased COX-2 gene expression, which may play an important role in UV carcinogenesis, and reduces UVB-induced mouse epidermal apoptosis [12,13]. However, seldom report about the effect of COX-2 on UVB-induced HaCaT cells apoptosis.

The incidence of UVB-related skin problems and the interest in protecting the skin from the harmful effects of UVB are increasing.

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In recent years, naturally occurring compounds have gained considerable attention as protective agents. However, many antioxidants mainly come from terrestrial herbs and plants, we seldom read the reports of polypeptides as antioxidants, especially for those from marine products. Polypeptide from *Chlamys farreri* (PCF), Mr = 879 kDa, is a novel marine active material isolated from gonochoric Chinese scallop *C. farreri* applying modern marine life engineering (Appl:00111426.9, China). Studies from our laboratory have indicated that PCF could inhibit HaCaT cells apoptosis after UVB exposure [14]. In this study, we imitated the apoptotic model of UVB-induced HaCaT cells and investigated the possibility that PCF inhibited UVB-induced apoptosis in HaCaT cells through effects on ROS, NF-κB and COX-2.

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, stored at 4 °C. Cell culture materials were purchased from Gibco-BRL. ROS scavenger NAC and NF-κB inhibitor MG132 were obtained from Sigma. Anti-SOD1 and anti-COX-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Anti-β-actin and anti-phospho-NF-κB/p65 antibodies were purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China) and Cell Signaling Technology TM (Beverly, MA, USA), respectively. Horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primers of CAT, GPx, COX-2 and GAPDH were designed and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). All the other chemicals used were of the highest grade commercially available.

2.2. Cell culture and UVB treatment

The human keratinocyte cell line HaCaT, was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. The cells were randomly divided into several groups including control group (normal cultural cells), model group (UVB-irradiated cells), PCF group (PCF pretreated and then UVB-irradiated cells) and the inhibitor group (the corresponding inhibitor pretreated and then UVB-irradiated cells). When cells were grown to 80–90% confluence, UVB exposure was performed with UVB lamps (Beijing Normal University, China), the wavelength range of UVB lamps was 290–320 nm with a peak wavelength at 297 nm. The irradiation intensity was monitored by a UVB radiometer was 11 mw/cm^2 (Beijing Normal University, China). And the dosage irradiated to these cells was 20 mJ/cm^2 after 30 min. PCF, NAC and MG132 were added in medium 2, 2 and 1 h, respectively, before irradiation. During irradiation, medium was discarded and cells were placed in PBS. After irradiation, the cells were cultured in compete culture medium again for the appropriate time.

2.3. DNA fragmentation assay

DNA fragmentation is a marker of cell apoptosis. Briefly, the HaCaT cells were washed with D-Hanks solution and harvested at 18 h after UVB irradiation. Then cell pellets were incubated in cell lysis buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.5% SDS, 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). DNA was precipitated with sodium acetate and absolute ethanol at −20 °C overnight, and then washed with 70% ethanol. 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, stained with ethidium bromide and visualized under UV light.

2.4. Detection of ROS by electron spins resonance (ESR)

After UVB treatment, the HaCaT cells were harvested. Briefly, 1×10^8 cells were homogenized on ice in 2 mL of 0.2 M Phosphate Hepes Buffer (0.038 M NaH_2PO_4, 0.162 M Na_2HPO_4, 0.01 mM EDTA, 10 mM HEPES, 0.32 M Glucopyranosid, 5 mM Mercaptoethanol, 10 mM PBN, 0.5% TW80, 2 mM DETAPAC), then centrifuged at 12,000*g* at 4 °C for 10 min. After centrifugation the supernatant was collected in 1.4 mL, then added in 30μL 0.5 M $Na_2S_2O_4$, 0.6 M DETC, 10 mM L-Arg, 0.3 M $FeSO_4$, respectively, and then incubated at 37 °C for 30 min. Placed the mixed liquor on ice quickly, and then added in 300 μL Acetoacetate and vortex 15 s. After centrifugation (12,000*g* for 8 min) at 4 °C, the supernatant was collected. The ROS was determined by Electron spin resonance (ESR) (Central Magnetic Field 3385G, Sweep Width 400G, Power 20 mW, magnification 4×10^5).

2.5. Western blot analysis

SOD1, COX-2, p-NF-κB/p65 were analyzed by Western blot. After UVB treatment, the HaCaT cells were harvested. Briefly, total cellular protein was extracted in ice-cold lysis buffer (20 mM Tris–HC1, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/l leupeptin, 1 mM PMSF). After centrifugation at 10,000*g* at 4 °C for 10 min, the protein concentration was measured with the BCA protein assay kit (Beyotime Biotechnology, China). Protein (40 μg) were resolved on a 10% SDS–PAGE and electroblotted to nitrocellulose membranes. The membranes were then blocked with 5% BSA in TBST at room temperature for 2 h and subsequently incubated with rabbit antibodies against SOD1 (1:200), COX-2 (1:200), p-NF-κB/p65 (1:000) and β-actin (1:400) overnight at 4 °C. Goat anti-rabbit secondary antibodies were diluted at 1:400 in 5% BSA/ TBST and were incubated with membranes for 40 min at 37 °C. Protein bands were visualized using the diamino-benzidine detection kit (Boster Biotechnology, Wuhan, China). The densities of sample bands were analyzed with Quantity One analysis software.

2.6. Detection of CAT, GPx and COX-2 by RT-PCR

Total RNA was extracted from HaCaT cells according to the manufacturer's protocol with Trizol reagent (Takara, China). First-strand cDNA was prepared by incubation of 1 μg total RNA with reverse transcriptase and oligo (dT) at 42 °C for 15 min. Then 2 μL of the reaction products was amplified by PCR with 2.5 U of *Taq* polymerase (Promega, Madison, WI, USA).The sequences of specific oligonucleotide primers were as follows: CTA TCC TGA CAC TCA CCG CCA T and TTC TTG ACC GCT TTC TTC TGG A for human CAT (372 bp); TGA ATT CCC TCA AGT ACG TCC G and AAA GTT CCA GGC AAC ATC GTT G for human GPx (215 bp); GTC CCT GAG CAT CTA CGG TTT G and CCC ATT CAG GAT GCT CCT GTT for human COX-2 (136 bp) (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 5 min and 30 cycles at 95 °C for 30 s and 58 °C for 30 s and 72 °C for 30 s for GPx and CAT; or 35 cycles at 94 °C for 40 s and 60 °C for 40 s and 72 °C and 40 s for COX-2 and a final

extension step was taken at 72 °C for 10 min. The PCR products were mixed with 2 ml of gel loading buffer, electrophoreses through a 1% agarose gel and visualized by ethidium–bromide staining. The intensity of each band was calibrated to the standard molecular marker on the same gel and then was normalized against the intensity of GAPDH.

2.7. Statistical analysis

Statistical analysis was performed with one-way ANONA, followed by the Bonferroni test using Origin7.5 (OriginLab Corporation, Northampton, MA, USA). The difference were considered significant if $P < 0.05$.

3. Results

3.1. Inhibitory effects of PCF, NAC and MG132 on HaCaT cells apoptosis induced by UVB irradiation

DNA ladder is a biochemical hallmark of apoptotic cell death, so internucleosomal DNA fragmentation in HaCaT cells was detected by using an agarose gel electrophoresis. In fact, DNA laddering determinates the amount of DNA degraded upon treatment of cells with certain agents such as UV radiation. Based on our prior studies with UVB and HaCaT cells [14], HaCaT cells were exposed to a UVB dose of 20 mJ/cm^2, which caused an increase in DNA fragmentation compared with control cells, pretreatment with 5.68, 1.42 mM PCF for 2 h prior to irradiation markedly attenuated UVB-induced DNA fragmentation (Fig. 1, lanes 2–4), thus supporting PCF protection against UVB. In addition, in NAC and MG132 treated cells, UVB did not induce DNA fragmentation (Fig. 1, lanes 5 and 6). Above-mentioned results indicate that UVB-induced apoptosis in HaCaT cells is mediated by ROS and NF-κB, PCF effectively inhibits apoptosis of HaCaT cells after UVB exposure.

3.2. Inhibitory effects of PCF and NAC on UVB-induced ROS generation in HaCaT cells

Many studies suggested that UV radiation can induce change in the intracellular ROS level [15]. To examine the effects of UVB-induced ROS on apoptosis in more detail, a kinetic analysis of intracellular ROS formation was performed using Electron spins resonance (ESR) by detecting 0, 1, 3, 12, 18, 24, 48 h post-irradiation. After exposure, two distinct levels of ROS were generated following irradiation: a pronounced increase observed early in 1 h that peaked in 3 h and then gradually declined in a time-dependent manner around 18 h, and a small late increase in 24 h, and then decreased rapidly with subsequent recovery to baseline levels in 48 h after radiation (Fig. 2A). In order to elucidate the molecular basis of the anti-apoptotic effect of PCF, we determined whether PCF exerts an inhibitive effect on UVB-induced ROS production. As our prior study has demonstrated that the concentration of PCF in IC50 was 2.84 mM, so we selected this concentration to observe the effect of PCF on UVB-induced generation of ROS. The data showed that PCF significantly decreased the generation of ROS at any of the evaluated times except in 3 h post-irradiation. The inhibition ratio were about 30% and 75% respectively, pretreatment of NAC also decreased the ROS levels, and the inhibitory effect was lower than the effect of PCF in 3 h, as shown in Fig. 2B and C.

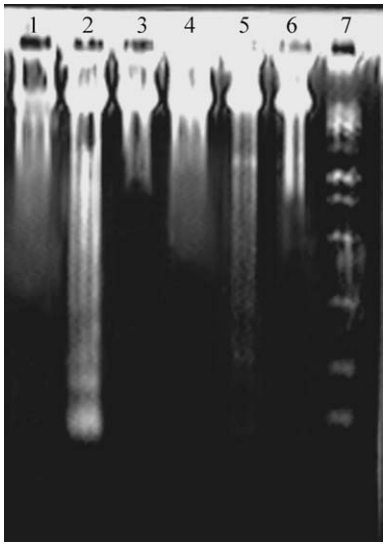

Fig. 1. Effect of PCF, NAC and MG132 on UVB-induced HaCaT cells apoptosis by DNA fragmentation assay. Lane 1: control; lane 2: cells were irradiated with 20 mJ/cm^2 UVB; lanes 3–6: cells were pretreated with 1.42, 5.69 mM PCF, 5 mM NAC and 0.2 μM MG132 for 2, 2, 2 h and 1 h prior to UVB irradiation, respectively; lane 7: standard base pair marker (2000, 1000, 750, 500, 250, 100 bp).

3.3. Effects of PCF on the expression of Cu, Zn-SOD in UVB-induced HaCaT cells

Under normal physiological conditions, ROS are rapidly eliminated by antioxidant enzymes, including superoxide dismutases (SODs), catalase, and glutathione peroxidase (GPx) [16]. SOD belongs to major antioxidant enzymes that contribute to the homeostasis of oxygen radicals in the epidermis and thus critically participates in the control of senescence and tumor generation [17]. It has been recently reported that two isozymes of SOD, namely copper–zinc SOD (Cu–Zn SOD or SOD1) and manganese SOD (Mn-SOD or SOD2), exist in mammalian cells and that the two enzymes play different roles in living systems, the former participate in an early phase and the latter in a late phase defense mechanism directed against oxidant cytotoxicity through UVB irradiation [17]. As we have demonstrated that UVB-induced HaCaT cells occurred in 18 h, so we evaluated the expression of Cu, Zn-SOD in UVB-induced HaCaT cells. As shown by the Western blot analysis (Fig. 3), exposure of HaCaT cells to UVB radiation significantly decreased the Cu, Zn-SOD protein level in 18 h post-irradiation, pretreatment of HaCaT cells with 1.42–5.69 mM PCF before UVB exposure strongly prevented the depletion of Cu, Zn-SOD in a dose-dependent manner as shown in Fig. 3.

3.4. Effects of PCF on the expression of CAT in UVB-induced HaCaT cells

Catalase is another endogenous antioxidant enzyme involved in the catalytic conversion of H_2O_2 to oxygen and water and thus decreases the level of oxidative stress. As shown in Fig. 4, the irradiation of HaCaT cells with UVB resulted in reduction of catalase in mRNA levels in 18 h after irradiation compared to non-UVB-exposed HaCaT cells, whereas pretreatment of HaCaT cells with 1.42–5.69 mM PCF enhanced the expression of catalase in a dose-dependent manner as shown in Fig. 4.

3.5. Effects of PCF on the expression of GPx in UVB-induced HaCaT cells

GPX, a selenoenzyme located in the cytoplasm and mitochondria that inactivates hydrogen peroxide as well as a wide range of lipid hydroperoxides, works in tandem with CAT to scavenge endogenous peroxides [18]. Our data demonstrated that UVB radiation significantly decreased the mRNA levels of GPx compared to non-UVB-exposed normal HaCaT cells (Fig. 4). Pretreatment of HaCaT cells with 1.42–5.69 mM PCF before UVB exposure prevented the depletion of GPx in a dose-dependent manner as shown in Fig. 5.

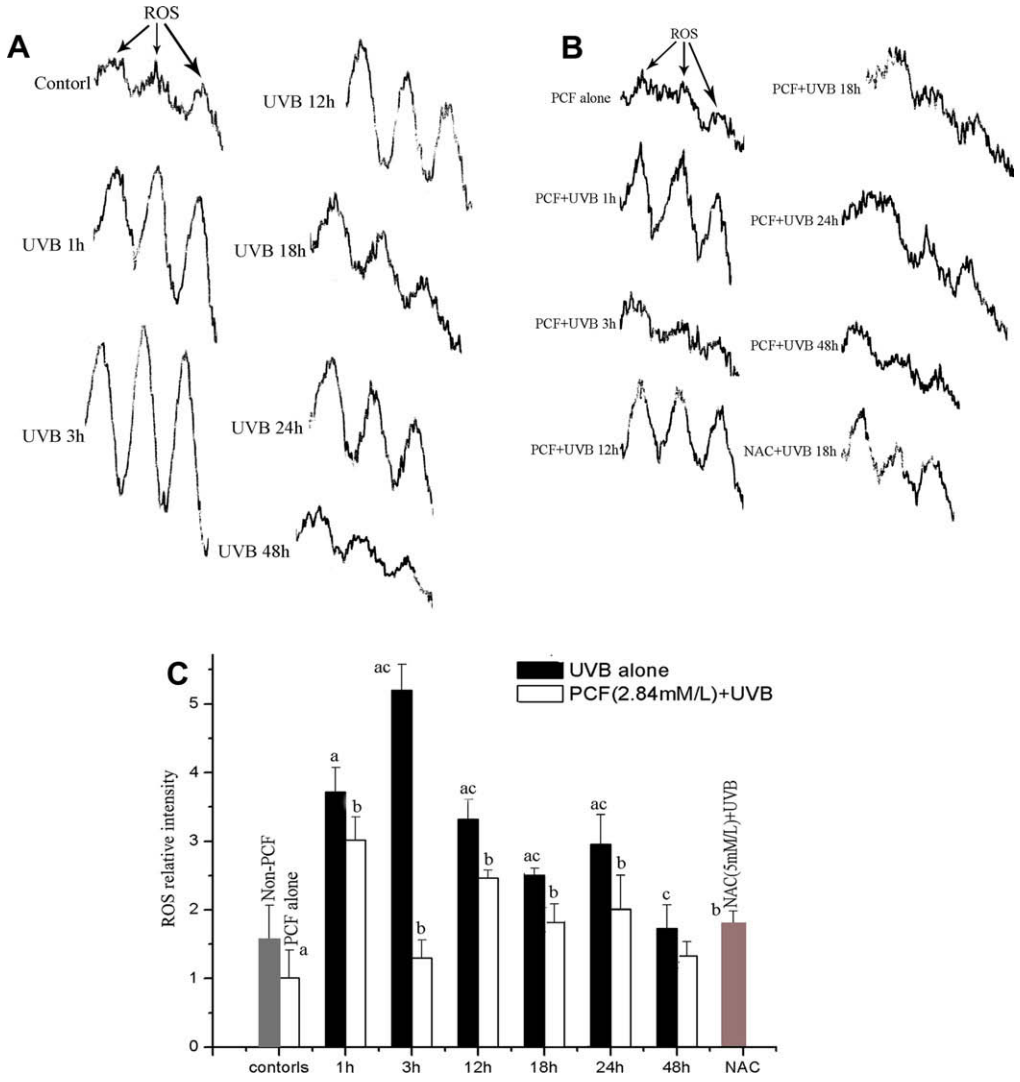

Fig. 2. Effect of PCF and NAC on UVB-induced generation of ROS in HaCaT cells. (A, B), Ctr – control; UVB – cells were irradiated with 20 mJ/cm^2 UVB; UVB + PCF, NAC–cells were pretreated with 2.84 mM PCF, 5 mM NAC for 2 h prior to UVB irradiation, respectively. After irradiation, cells were incubated at different times (1, 3, 12, 18, 24, 48 h), and ROS were detected by electron spin resonance spectrometry. Results shown are representative of three independent experiments. (C) Quantification of (A and B) results. $^{a}P < 0.01$ vs. control; $^{b}P < 0.01$ vs. UVB in the same group; $^{c}P < 0.01$ vs. with the forward group.

3.6. Effects of PCF and NAC on the activation of NF-κB/p65 in UVB-induced HaCaT cells

Numerous studies indicated that oxidative stress induced by increased generation of ROS is involved in the activation of NF-κB, which could promote the translocation of NF-κB [19]. The efficient transcriptional activation of NF-κB depends on the phosphorylation of its active subunit p65/RelA, particularly at serine 536 residue [19]. To investigate whether the protective effect of PCF on UVB-induced apoptosis is related to NF-κB/p65, we treated HaCaT cells with different concentrations of PCF before UVB exposure and then tested for phosphorylated NF-κB/p65. As shown in Fig. 6, UVB radiation induced the phosphorylation of at Ser (536) so as to enhance the activation of NF-κB, Western blot and subsequent measurement of the intensity of bands relative to β-actin indicated that treatment with 1.42–5.69 mM PCF markedly inhibited UVB-induced phosphorylation of NF-κB/p65 in a dose-dependent manner (Fig. 6). Similar to the suppression of PCF on UVB-induced phosphorylation of NF-κB/p65, pretreatment of NAC significantly inhibited UVB-induced phosphorylation of NF-κB/p65 (Fig. 6). Our data suggested that ROS could induce the trans-activation of NF-κB, thus to increase the sensitivity of apoptosis in UVB-induced HaCaT cells, PCF exerts its protective effect via suppression of the trans-activation of NF-κB.

3.7. Effects of PCF on the expression of COX-2 in UVB-induced HaCaT cells

Cyclooxygenase (COX) plays a major role in the UVB-induced ROS production and activation of NF-κB in HaCaT cells [9], which have two isoforms of this enzyme: COX-1 and COX-2; COX-1 is constitutively expressed in most tissues, whereas COX-2 is inducible by a variety of tumor-promoting agents [20]. An inappropriate over-expression of COX-2 is implicated in carcinogenesis. So we firstly study the effect of UVB irradiation on the expression of COX-2, HaCaT cells were exposed to UVB at doses of 20 mJ/cm^2, and then cells were harvested at different times after UVB irradiation. As shown in Fig. 7A, increased expression of COX-2 was clearly visualized at 6 h, following the markedly increased expression at 18 h after UVB (20 mJ/cm^2) irradiation, and then declined gradually; the expression at 24 h was similar to 1 h after UVB (20 mJ/cm^2) irradiation. Then we investigated effects of PCF on

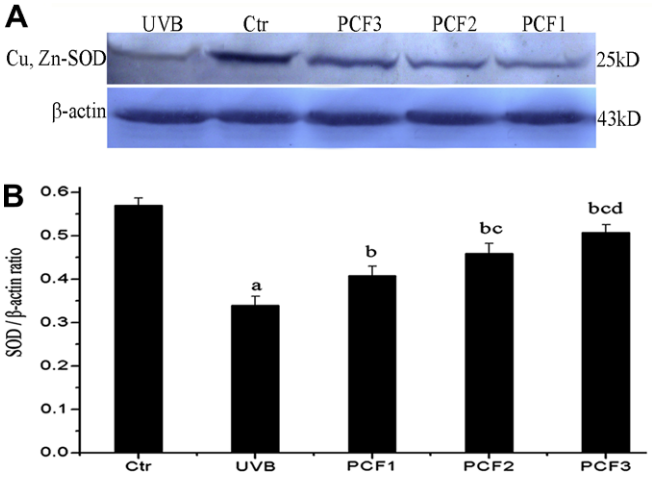

Fig. 3. Effect of PCF on UVB-induced Cu, Zn-SOD protein expression in HaCaT cells. (A) Ctr – control; UVB – cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3, cells were pretreated with 1.42, 2.84, 5.69 mM PCF for 2 h prior to UVB irradiation, respectively. After irradiation, cells were incubated for 18 h, and Cu, Zn-SOD was detected by Western blot. Results shown are representative of three independent experiments. (B) Quantification of (A) results. Results were expressed as the ratio of expression level of Cu, Zn-SOD 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.

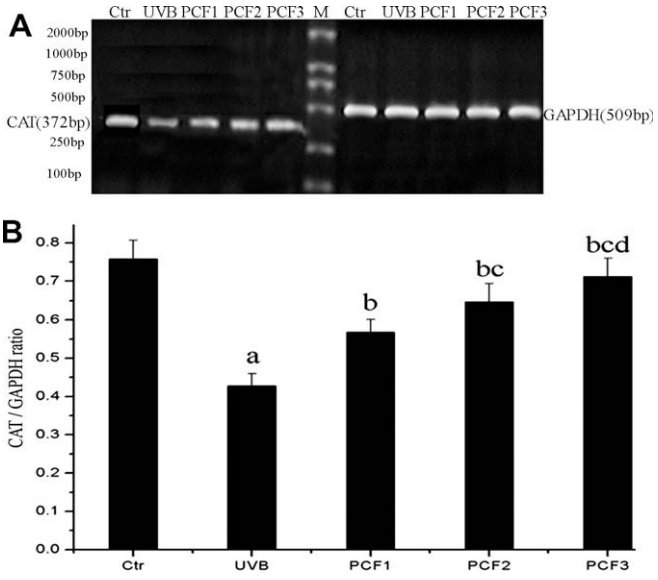

Fig. 4. Effect of PCF on UVB-induced CAT mRNA expression in HaCaT cells. (A) Ctr – control; UVB – cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3, cells were pretreated with 1.42, 2.84, 5.69 mM PCF for 2 h prior to UVB irradiation, respectively. After irradiation, cells were incubated for 18 h, and CAT was detected by RT-PCR. Results shown are representative of three independent experiments. (B) Quantification of (A) results. Results were expressed as the ratio of expression level of CAT 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.

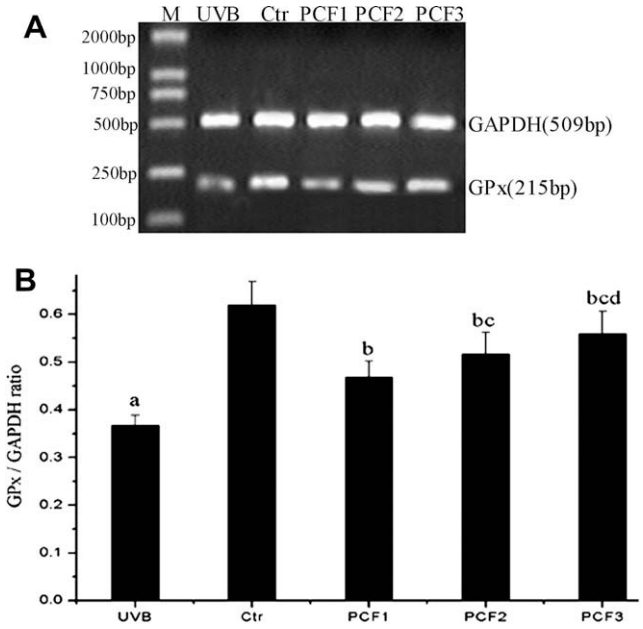

Fig. 5. Effect of PCF on UVB-induced GPx mRNA expression in HaCaT cells. (A) Ctr – control; UVB – cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3, cells were pretreated with 1.42, 2.84, 5.69 mM PCF for 2 h prior to UVB irradiation, respectively. After irradiation, cells were incubated for 18 h, and GPx was detected by RT-PCR. Results shown are representative of three independent experiments. (B) Quantification of (A) results. Results were expressed as the ratio of expression level of GPx 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.

COX-2 expression in UVB-induced HaCaT cells at 18 h after irradiation for Western blot. As shown in Fig. 7B and C, up-regulation of COX-2 by UVB was markedly decreased in a concentration-dependent manner.

We also examined the expression of COX-2 mRNA by RT-PCR analysis for the further identification. We performed RT-PCR analysis using specific COX-2 primers to determine whether up-regulation of COX-2 is regulated at the level of transcription. In agreement with Western, increased expression of COX-2 mRNA was clearly visualized at 18 h after UVB (20 mJ/cm^2) irradiation compared with untreated cells (Fig. 8), indicating that the up-regulation of COX-2 by UVB in HaCaT cells is largely due to increased synthesis of COX-2 mRNA. To investigate whether PCF attenuates COX-2 expression by UVB at transcriptional level, we performed RT-PCR analysis using COX-2 specific primers. As shown in Fig. 8, increased expression of COX-2 mRNA by UVB was clearly inhibited in the presence of PCF in a dose-dependent manner. These results inferred that PCF inhibited UVB-induced apoptosis through its effects on COX-2.

4. Discussion

Apoptosis is a tightly regulated form of cell death and a multifactor-related process, including gene expression and mutation. UVB irradiation has been shown to be a particularly potent inducer of apoptosis [21]. Kulms et al. indicated that DNA damage, death receptor activation, and ROS formation all contribute to UVB-induced apoptosis in different ways [22]. Despite rapid progress in understanding apoptosis, the role of ROS production in cell death and its relationships with other apoptotic events are still unclear. UVB-induced apoptosis is a highly complex process involving the extrinsic and intrinsic pathways, but it is unclear how these pathways are interrelated. In this study, UV irradiation induced the formulation of DNA laddering obviously, indicating that we successfully imitated the UVB irradiation induced apoptosis model of HaCaT cells. Pretreatment with PCF inhibited HaCaT cells apoptosis dose-dependently, which were consistent with our previous study. We also observed that NAC and MG132 protected the cells apoptosis, suggesting that ROS and NF-κB play important roles in cellular apoptosis after UV irradiation in HaCaT cells. Therefore, we further investigated the relationship between UV irradiation and signal pathways in HaCaT cells; and then we tried to determine the protective effects of PCF on preventing HaCaT cells from apoptosis induced by UVB.

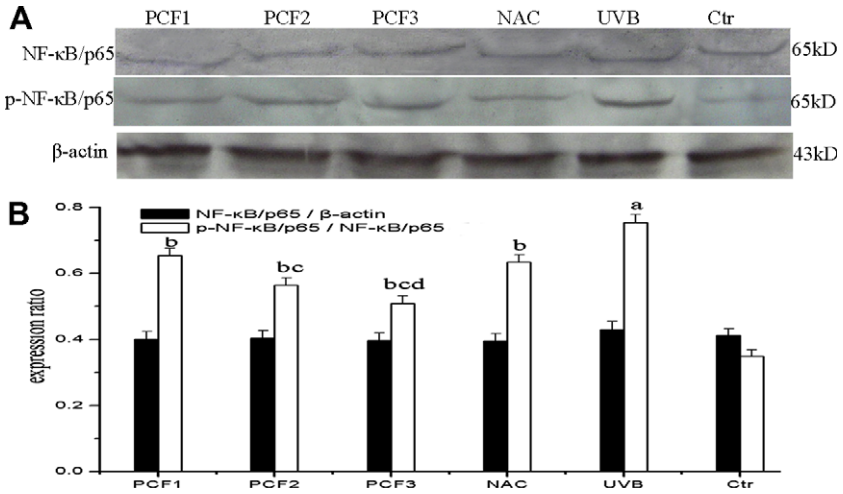

Fig. 6. Effect of PCF on UVB-induced p-NF-κB/p65 protein expression in HaCaT cells. (A) Ctr – control; UVB – cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3,NAC, cells were pretreated with 1.42, 2.84, 5.69 mM PCF, 5 mM NAC for 2 h prior to UVB irradiation, respectively. After irradiation, cells were incubated for 3 h, and p-NF-κB/p65 was detected by Western blot. Results shown are representative of three independent experiments. (B) Quantification of (A) results. Results were expressed as the ratio of expression level of phosphorylated NF-κB/p65 or total NF-κB/p65 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.

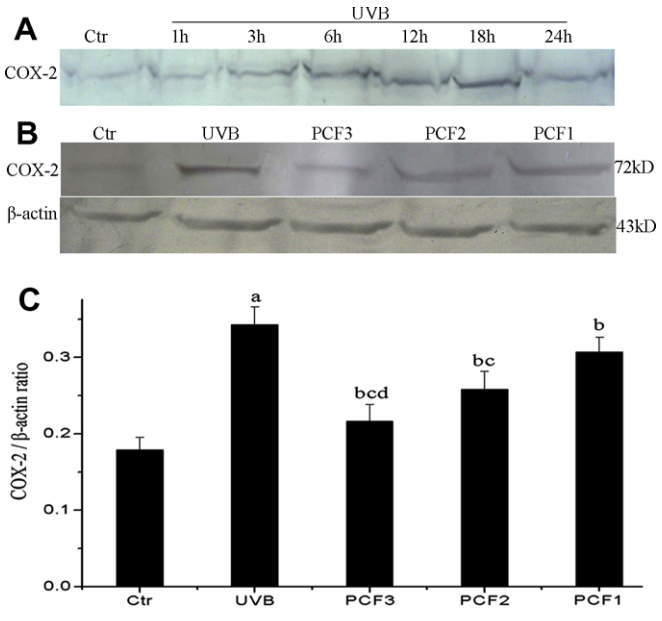

Fig. 7. Effect of PCF on UVB-induced COX-2 protein expression in HaCaT cells. (A) Ctr – control; UVB – cells were irradiated with 20 mJ/cm^2 UVB. After irradiation, cells were harvested at indicated time (1, 3, 6, 12, 18, 24 h), and COX-2 was detected by Western blot. B, PCF1–3, cells were pretreated with 1.42, 2.84, 5.69 mM PCF for 2 h prior to UVB irradiation, respectively. After irradiation, cells were incubated for 18 h, and COX-2 was detected by Western blot. Results shown are representative of three independent experiments. (C) Quantification of (B) results. Results were expressed as the ratio of expression level of COX-2 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.

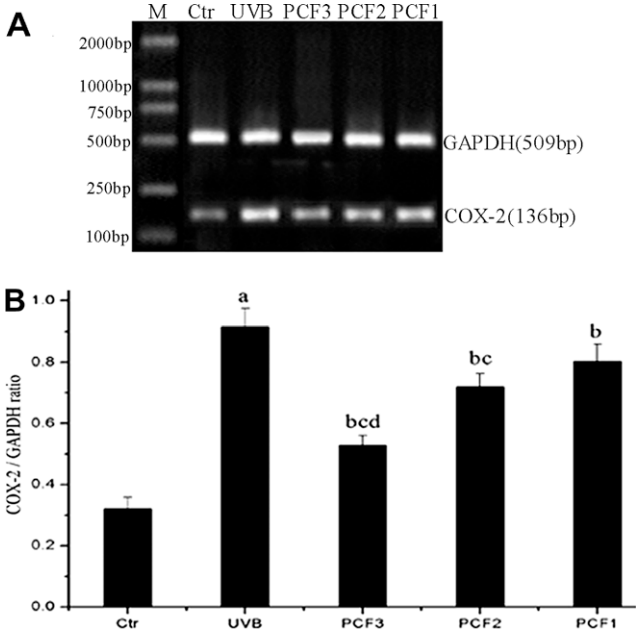

Fig. 8. Effect of PCF on UVB-induced COX-2 mRNA expression in HaCaT cells. (A) Ctr – control; UVB – cells were irradiated with 20 mJ/cm^2 UVB; PCF1–3, cells were pretreated with 1.42, 2.84, 5.69 mM PCF for 2 h prior to UVB irradiation, respectively. After irradiation, cells were incubated for 18 h, and COX-2 was detected by RT-PCR. Results shown are representative of three independent experiments. (B) Quantification of (A) results. Results were expressed as the ratio of expression level of COX-2 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.

UVB radiation is known to be a potent inducer of significant amounts of ROS production, which have been implicated in skin inflammation and cancer [23]. Several studies have demonstrated that an increase in antioxidant defense systems can reduce the deleterious effects of UV-induced ROS [24,25]. However, when the antioxidant response is overwhelmed by considerable amounts of ROS, oxidation of proteins, DNA and lipids occurs, leading to deleterious effects on cells and therefore, contributing to UVB-induced apoptosis [26]. Lee et al. confirmed that eriodic- tyol protects keratinocytes from UV induced apoptosis via the suppression of intracellular ROS generation [27]. To determine the impotent role of ROS played in UVB-induced HaCaT cells apoptosis fatherly, we examined the ROS levels induced by UVB irradiation at several times by ESR. ESR is a direct measurement of free radical including hydroxyl, alkyl, and superoxide radicals, which also called ROS. Our resulted demonstrated that UVB irradiation induced ROS production at two distinct stages; a pronounced increase observed early in 3 h, then declined very rapidly time-dependently, the lowest was in 18 h then a small late increase in 24 h and was no longer significantly different from controls at 48 h post-UVB, but generally speaking, it strongly increased the ROS levels in all times;

The source of the initial phase increase in ROS levels is not definitely known, but our previous study found the reduction in apoptosis, accompanied by a collapse of mitochondrial inner transmembrane potential, cytochrome *c* release and a decrease in caspase-9 activation, suggests that mitochondria could be the main source of this ROS generation [14,28,29]. Then the kinetic process of intracellular ROS formation remains to be determined, but we think it may be associated with the activation of the antioxidant enzymes; which can eliminate the ROS rapidly, so the ROS gradually decline. When the cleared abilities of them are saturated, they could not eliminate ROS any more, it occurs the second slight increase phase in ROS levels. Pretreatment with NAC not only deceased the generation of ROS, but also abolished UVB-induced apoptosis. The data indicated that ROS occupied an essential role in UVB-induced HaCaT cells apoptosis especially in the early phase of apoptosis, and the first increase in ROS production has a major role in UVB-induced apoptosis. Two phases of ROS production were also reported in a model of apoptosis induced γ-irradiation in the IM-9 cell line [30].

Balaban et al. indicated that an impairment in the function of antioxidant enzymes results in the accumulation of ROS [16]. Antioxidant enzymes function cooperatively, and any change in one of them may affect the equilibrium state of oxidative stress or ROS [31]. Antioxidant enzymes including CAT, SOD and GPx act in concert to protect cellular components from damage by ROS, which represents the primary line of defense [32]. SOD is the most important enzymatic anti-oxidant to protect cells from UVB damage [3], which catalyses dismutation of two superoxide anions to form hydrogen peroxide and oxygen [33]. GPx is a selenoprotein that catalyses the conversion of UV-induced H_2O_2 into water and molecular oxygen using GSH as a cosubstrate, which considered to be the most important antioxidant defense system in the skin [34]. CAT is one of the primary antioxidative enzymes in cells which catalyses the conversion of H_2O_2 into water and molecular oxygen thus reduces the damaging effects of H_2O_2, it is a major determinant of cellular resistance to hydrogen peroxide toxicity [34]. Our previous study had demonstrated that UVB radiation strongly reduced the activity of these antioxidative enzymes [35]. In this study, we observed that UVB radiation significantly decreased the Cu, Zn-SOD protein level and CAT, GPx mRNA levels protein level at 18 h post-irradiation, these decrease were probably due to irreversible oxidation of the enzyme and neutralization the generation of ROS as the formation of ROS was lowest in 18 h. The reduced activity of these antioxidative enzymes may be caused by the decreased expression in protein and mRNA levels, respectively. All these indicated that antioxidative enzymes could protect cells apoptosis induced by UVB.

Taken together, the evidence to date suggests that the increasing generation of ROS and the decreasing expression of antioxidative enzymes (Cu, Zn-SOD, CAT and GPx) induced by UVB irradiation caused a pro-oxidant/antioxidant disequilibrium, thus resulted in an apoptosis of HaCaT cells. This finding is consistent with observations reported by Alena et al. demonstrating that UVB provokes free radical production and induces a significant decrease in skin antioxidants, impairing the skin's ability to protect itself against the free radicals generated after sunlight exposure [2]. Agents that scavenge ROS could exert its photoprotective effects. Our previous study has showed that PCF could decrease the generation of ROS, but we never see the effect of PCF on the kinetic analysis of intracellular ROS formation. In our experiment, PCF could inhibit the generation of ROS in any evaluated times, it could last 48 h. The inhibitory effect was strongest in 3 h and in other times the effects were similar. Meanwhile PCF enhanced the expression of antioxidative enzymes, indicating that PCF could suppress HaCaT cells apoptosis. As PCF could scavenge free radicals, activated or enhanced the antioxidant defenses of the target cells to produce a marked protective effect, which may also be considered as one of the possible mechanisms of chemoprevention of photocarcinogenesis.

A number of studies have confirmed that ROS significantly contribute to UV-induced signal transduction such as MAPKs, NF-κB and AP-1. NF-κB is a pleiotropic transcriptional activator, which is a sensitive transcriptional factor for free radicals and activates multiple target genes involved in immune and inflammatory responses and in cell growth control [36].Because cellular consequences of activating the NF-κB pathway are complex, so the activation of NF-κB can have both pro- and antiapoptotic effects in various cell types [37]. However, UVB-induced activation of NF-κB could promote UVB-induced HaCaT cells apoptosis [11]. As the phosphorylation of its active subunit p65/RelA at serine 536 residue determinates the transcriptional activation of NF-κB, So it was of interest to examine the activation mechanism and the protective effect of PCF on UVB-induced apoptosis through NF-κB. In our experiment, we demonstrated that NAC effectively inhibited phosphorylation of NF-κB/p65 induced by UVB and PCF attenuated NF-κB activation dose-dependently. These results suggest that PCF inhibited UVB-induced apoptosis through its effects on NF-κB, the inhibitory effect may partly depend on its property as a ROS scavenger.

Cyclooxygenase-2 (COX-2), a key enzyme in the prostaglandin biosynthesis, plays important roles in the development of carcinogenesis as well as inflammation in UVB irradiated Skin, which has been recognized as a molecular target for many anti-inflammatory as well as chemopreventive agents [38]. Joydeb et al. has reported that inhibition the activation of NF-κB by an IKK inhibitor Bay 11-7082 could abrogate COX-2 expression, suggesting the important role of NF-κB in regulating COX-2 expression [39]. Since NF-κB is a known positive regulator of COX-2 transcription and we found that PCF treatment inhibited activity in UVB-induced HaCaT cells, we attempted to determine the effect of PCF on UVB-induced expression of COX-2 transcription in HaCaT cells. In this study, we observed that UVB irradiation strongly induced the expression of COX-2, but was later than the activation of NF-κB, pretreatment with PCF significantly inhibited UVB-induced the expression of COX-2 both at protein and mRNA levels in a dose-dependent manner. These data indicated that PCF suppressed UVB-induced COX-2 expression; the inhibition effect may via diminishing the activation of upstream molecule NF-κB. Considering NF-κB as potential molecular links between apoptosis and the contributory role of aberrantly expressed COX-2 in tumor promotion [38], the present study provides the molecular mechanisms underlying previously reported chemopreventive effects of PCF on UVB-induced HaCaT cells apoptosis.

This paper reports new findings about the additional functions of the PCF in response to UVB irradiation. In conclusion, UVB caused apoptotic cell death by increasing ROS levels, decreasing expression of antioxidative enzymes and induced NF-κB activation and COX-2 expression. We found that PCF could protect HaCaT cells from damage by UV irradiation via scavenging ROS and increasing the expression of antioxidative enzymes as an anti-oxidant to block the signal pathway. Present observations provide new insight into the protective functions of PCF involved in the process of apoptosis in HaCaT cells induced by UVB. These results show that PCF is a good preventive regimen against UVB radiation, which could be optimized as a prophylactic agent to prevent the formation of sunburn cells and protect the natural barrier function of the skin.

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