



## Polypeptide from *Chlamys farreri* modulates UVB-induced activation of NF- $\kappa$ B signaling pathway and protection HaCaT cells from apoptosis

Xiaojin Liu<sup>a</sup>, Zhengyang Zhang<sup>a</sup>, Ping Li<sup>b</sup>, Li Zhu<sup>b</sup>, Yuejun Wang<sup>c</sup>, Chunbo Wang<sup>a,\*</sup>

<sup>a</sup> Department of Pharmacology, Medical College of Qingdao University, Qingdao 266071, China

<sup>b</sup> Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, China

<sup>c</sup> Yellow Sea Fishery Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China

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### ABSTRACT

Polypeptide from *Chlamys farreri* (PCF) possesses strong antioxidant and photochemo-preventive properties. Our previous study has preliminarily demonstrated that PCF could reduce the intracellular reactive oxygen species (ROS) production and protect UVB-induced HaCaT cells apoptosis. But the anti-apoptotic effects of PCF on components of cell signaling pathways leading to gene expression has not been clearly established. In this study we determined whether PCF affords protection of HaCaT cells against UVB-mediated activation of nuclear factor kappa B signal pathway, which is involved in apoptosis. The result showed that pretreatment of UVB-induced HaCaT cells with PCF, ROS scavenger NAC and NF- $\kappa$ B inhibitor MG132 effectively suppressed the apoptosis of HaCaT cells. PCF inhibited UVB-induced activation and translocation of NF- $\kappa$ B/p65 to nucleus, which was mediated through inhibition of phosphorylation/degradation and decreasing mRNA expression of I $\kappa$ B $\alpha$  and also blocking activation of IKK $\alpha$  in a dose-dependent manner. Furthermore, we observed that NAC also inhibited UVB-induced activation of NF- $\kappa$ B/p65 through decreasing the degradation and phosphorylation of I $\kappa$ B $\alpha$ . We concluded that the activation of NF- $\kappa$ B signal pathway played an important role in UVB-induced apoptosis, and PCF likely exerted its anti-apoptotic effect in HaCaT cells through decreasing intracellular ROS level and modulating the NF- $\kappa$ B signaling pathway.

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### 1. Introduction

Ultraviolet (UV) light, mainly the UVB spectrum; can cause acute cutaneous inflammation, sunburn, accelerated skin ageing, cancer and immunosuppression [1,2]. The detrimental effects of ultraviolet B (UVB) irradiation have been connected with the UVB enhanced generation of reactive oxygen species (ROS), which have been suggested to play a role as second-messenger molecules in the signaling and regulation of gene expressions related to the biological effects elicited by UVB [3]. A number of studies have confirmed that UV significantly contributes to apoptosis of human keratinocytes in vivo and in vitro [4]. ROS induced by UV irradiation also play a very important role in apoptosis induction under both physiological and pathological conditions [5].

Nuclear factor-KappaB (NF- $\kappa$ B) is a pleiotropic transcriptional activator, which is sensitive to free radicals and activates multiple target genes [6]. It is also a key transcription regulator in keratinocytes [7]. Many studies suggested that UV irradiation causes an increase in ROS levels as well as oxidative damage to DNA, which is thought to

activate one components of the multiprotein I $\kappa$ B kinase complex which lead to I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B translocation so as to stimulate NF- $\kappa$ B activation [8–10]. The activation of NF- $\kappa$ B can induce a variety of cellular responses, including the induction of inflammation, cell proliferation, differentiation, or apoptosis; these divergent cell responses to NF- $\kappa$ B activation are defined by cell-specific and stimuli-specific mechanisms [11]. In most cases, NF- $\kappa$ B functions as a survival factor. But in HaCaT cells, because of the aberrant NF- $\kappa$ B activity increased the sensitivity of HaCaT cells to UVB-induced apoptosis [12]. It has been suggested that NF- $\kappa$ B plays an important role in cellular death after UV irradiation [13,14]. Additionally, many articles also indicated that UV-induced activation of NF- $\kappa$ B-dependent gene transactivation pathways is a critical event for the subsequent development of sunburn reactions in skin [15,16]; so particular focus was directed at this transcription factor.

In order to diminish the adverse effects of UV irradiation, considerable attention should be paid to the development and implementation of novel prevention approaches. Therefore, it is suggested that the topical application of radical scavengers and antioxidants like vitamin E, vitamin C and their derivatives may prevent premature skin ageing and indeed these substances are widely used [17]. However, these 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),

\* Corresponding author. 422 Room, Boya Building, Medical College, Qingdao University, 308 Ningxia Road, Qingdao 266071, China. Tel./fax: +86 532 83780029.

E-mail address: [lxj8208@sohu.com](mailto:lxj8208@sohu.com) (Chunbo Wang).

is a novel marine active material isolated from gonochoric Chinese scallop *C. farreri* applying modern marine life engineering (Appl.: 0011426.9, China). As a water-soluble octapeptide, PCF consists of Pro, Asn, Ser, Thr, Arg, Hyl, Cys and Gly, Mr=879 kDa. Previous studies from our laboratory have indicated that PCF has potential antioxidant activity and protective effect against UVB irradiation in human dermal fibroblasts and HaCaT cells [18,19], but its anti-apoptotic mechanisms were not completely understood.

Based on the important roles of ROS, NF- $\kappa$ B signal pathway in UV-induced apoptosis, in the present study, we imitated the apoptotic model of UVB-induced HaCaT cells and investigated the possibility that PCF protected UVB-induced apoptosis in HaCaT cells through ROS/NF- $\kappa$ B signaling pathway.

## 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. NF- $\kappa$ B inhibitor MG132 and ROS scavenger NAC were obtained from Sigma. Anti-IKK $\alpha$ , anti-I $\kappa$ B $\alpha$ , anti-phospho-I $\kappa$ B $\alpha$  and anti-NF- $\kappa$ B/p65 antibodies were purchased from Cell Signaling Technology™ (Beverly, MA, USA). The primers of I $\kappa$ B $\alpha$  and GAPDH were designed and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Anti- $\beta$ -actin and Anti-Lamin B antibodies were purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively; 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. 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<sub>2</sub> 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-un-irradiated or 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) and the dosage irradiated to these cells was 20 mJ/cm<sup>2</sup> after 30 min. PCF, NAC and MG132 were added in medium 2, 2 h 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. Hoechst 33258 staining

At 18 h after UVB treatment, cells were collected and fixed, washed twice with PBS and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Hoechst Staining Kit, Beyotime Biotechnology, China). Apoptotic features of cell death were determined by the staining of cell nuclei with the DNA-binding fluorochrome H33258 assessing chromatin condensation by fluorescence microscope (Leica DBI 4000 B) analyses. 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. Localization of p65 by indirect immunofluorescence

The NF- $\kappa$ B/p65 nuclear localization was detected by indirect immunofluorescence assay using fluorescence microscope (Leica DBI 4000 B). HaCaT cells grown on a glass slide in a six-well plate were exposed to UVB and incubated for the indicated time periods. According to the manufacturer's instructions (Immunol Fluorecence Staining Kit with FITC, Beyotime Biotechnology, China), HaCaT cells were washed with PBS and then were fixed with 4% ice-cold formaldehyde for 15 min. Polyclonal antibody to human NF- $\kappa$ B/p65 (1:100) was applied for overnight at 4 °C followed by 1 h incubation with fluorescein isothiocyanate conjugated goat anti-rabbit IgG (1:1000). The cells were observed under a fluorescence microscope.

### 2.5. Detection of I $\kappa$ B $\alpha$ by RT-PCR

Total RNA was extracted from HaCaT cells according to the manufacturer's protocol with Trizol reagent (Takara, China). The sequences of specific oligonucleotide primers were as follows: CTG TGA TCC TGA GCT CCG AGA C and GCC CCA CAC TTC AAC AGG AGT for human I $\kappa$ B $\alpha$  (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 94 °C for 1 min and 58 °C for 1 min and 72 °C for 1 min, and a final extension step was taken at 72 °C for 5 min. The PCR products were mixed with 2 mL of gel loading buffer, electrophoresed 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.

### 2.6. Cytosolic and nuclear extraction

After UVB treatment, the HaCaT cells were harvested. Briefly, 1 × 10<sup>6</sup> cells were pelleted, resuspended in 100  $\mu$ L of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.5 mM PMSF, 10  $\mu$ g/mL leupeptin, 10  $\mu$ g/mL aprotinin, 1 mmol·L<sup>-1</sup> Na<sub>3</sub>VO<sub>4</sub>, 1 mmol·L<sup>-1</sup> NaF), and kept on ice for 15 min. The cells were then lysed with 6.25  $\mu$ L 10% Nonidet P-40. After centrifugation (13,000 g for 5 min), the supernatant was collected as cytosolic extract, the nuclear pellet was resuspended in 12.5  $\mu$ L buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5  $\mu$ g/mL leupeptin, 5  $\mu$ g/mL aprotinin) on ice for 30 min. After centrifugation (13,000 g for 15 min) at 4 °C, the supernatant was collected as nuclear extract. The protein content of the extracts was determined by the BCA protein assay kit (Beyotime Biotechnology, China).

### 2.7. Western blot analysis

The phosphorylated I $\kappa$ B $\alpha$  (P-I $\kappa$ B $\alpha$ ), I $\kappa$ B $\alpha$ , NF- $\kappa$ B/p65 and IKK $\alpha$  were analyzed by Western blotting. At 3 h after treatment, the protein was extracted and quantitated as previously described in this study. 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 P-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , NF- $\kappa$ B/p65, IKK $\alpha$  or  $\beta$ -actin (dilution 1:1000) overnight at 4 °C, and then with peroxidase-conjugated secondary antibodies (dilution 1:500) for 2 h at room temperature. The bands were detected using the diaminobenzidine detection kit (Boster Biotechnology, Wuhan, China), and the

result was analyzed by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.8. Statistical analysis

Data were expressed as means  $\pm$  SD. Statistical analysis was performed with one-way ANOVA, 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 on HaCaT cells apoptosis induced by UVB irradiation

To determine whether UVB-induced HaCaT cells death were due to apoptosis, the morphologic changes in cell nuclei were examined by Hoechst 33258 stain. Based on our prior studies with UVB and HaCaT cells [19], HaCaT cells were exposed to a UVB dose of 20 mJ/cm<sup>2</sup>, marked morphologic changes in cell nuclei were observed (Fig. 1, lane 3) compared with the untreated control (Fig. 1, lane 2). In 2.84 mM PCF only treated cells, the apoptotic cells were markedly attenuated (Fig. 1, lane 1). When cells were pretreated with 5.68, 2.84 or 1.42 mM PCF for 2 h prior to irradiation, the number of apoptotic cells was concentration-dependently decreased (Fig. 1, lane 5–7), thus supporting PCF protection against UVB. In addition, in NAC treated cells, the number of apoptotic cells were also decreased obviously (Fig. 1, lane 4). Above-mentioned results indicate that UVB-induced apoptosis in HaCaT cells is mediated by ROS and PCF effectively

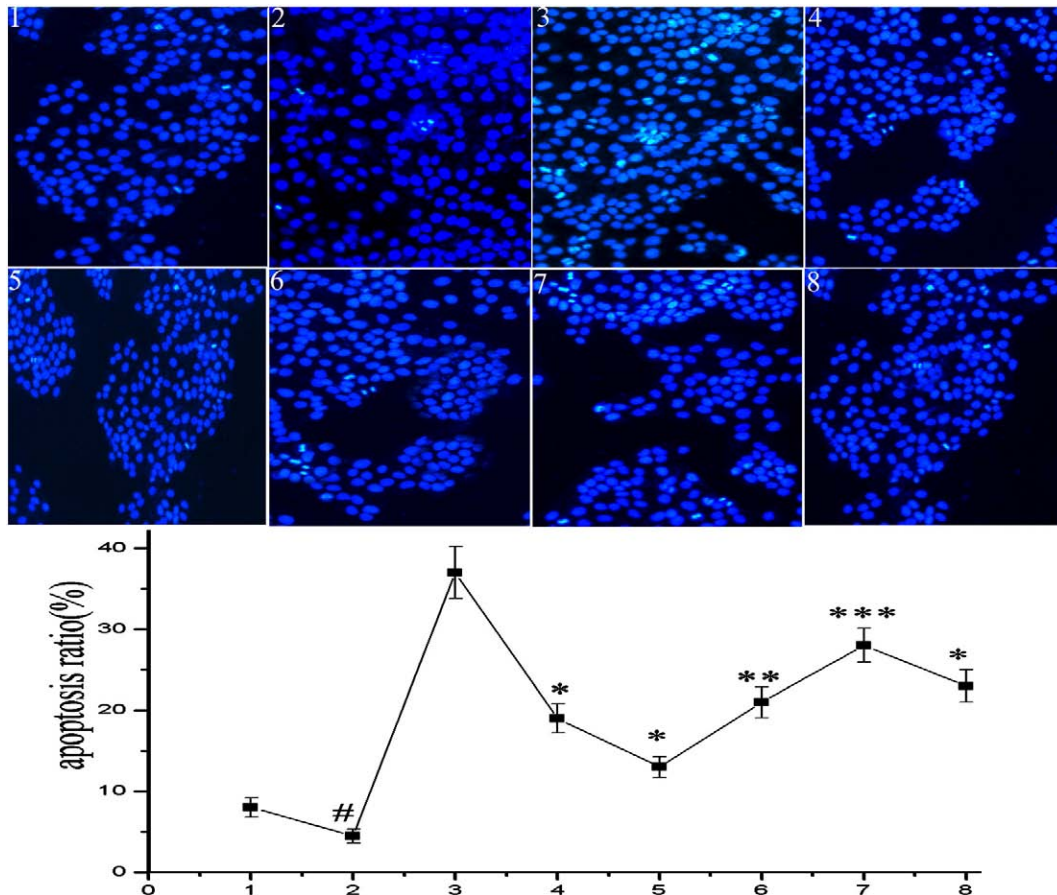
inhibits apoptosis of HaCaT cells after UVB exposure. Therefore in all further experiments, we used the optimum doses of UVB (20 mJ/cm<sup>2</sup>) and PCF (5.69, 2.84 or 1.42 mM) to define the photoprotective effects of PCF on UVB-induced oxidative stress.

### 3.2. Inhibition of NF- $\kappa$ B activity in HaCaT cells reduces UVB-induced apoptosis

NF- $\kappa$ B is a key transcription regulator in keratinocytes and plays a critically important role in both regulations of the cell cycle as well as influencing cell death pathways. However, as the cellular consequences of activating the NF- $\kappa$ B pathway are complex, so the nuclear translocation of NF- $\kappa$ B can have both pro- and anti-apoptotic effects [20]. Therefore, to address the contribution of NF- $\kappa$ B to induce apoptosis in HaCaT cells, we examined the effect of the specific NF- $\kappa$ B inhibitor MG132 on UVB-induced apoptosis. As shown in Fig. 1 (lane 8), preincubation of HaCaT cells with MG132 effectively prevented UVB induced apoptosis and significantly blocked NF- $\kappa$ B activation induced by UVB (Fig. 3). These results indicated that the NF- $\kappa$ B activity found in HaCaT cells contributed to the increased sensitivity of HaCaT cells to UVB-induced apoptosis. Thus, we were interested to see the possible molecular mechanisms occurring in UVB-induced NF- $\kappa$ B activation which induced the apoptosis of HaCaT cells.

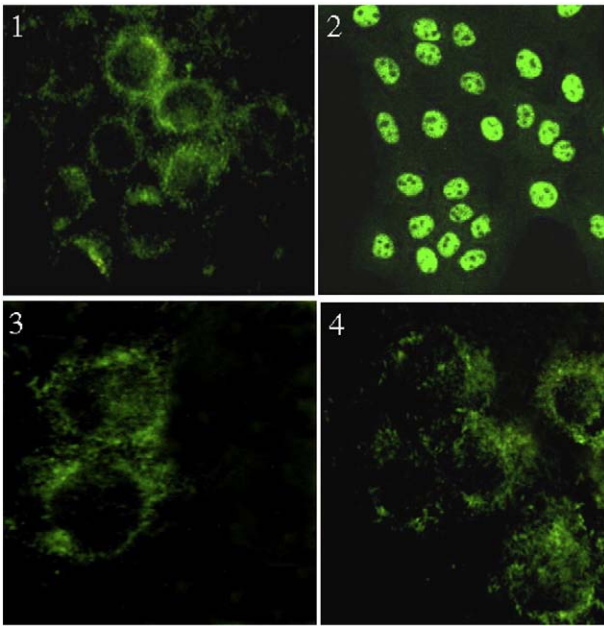
### 3.3. UVB radiation caused an enhanced activation and translocation of NF- $\kappa$ B/p65 to nucleus

UVB is considered to be the main component of the solar UV irradiation activating NF- $\kappa$ B in HaCaT keratinocytes [21]. Reactive



**Fig. 1.** Effect of PCF, MG132 and NAC on UVB induced HaCaT cells apoptosis by Hoechst 33258 staining. lane 1: Cells were pretreated with 2.84 mM PCF for 2 h; lanes 2: control; lane 3: Cells were irradiated with 20 mJ/cm<sup>2</sup> UVB; lanes 4–8: Cells were pretreated with 5 mM NAC, 5.69, 2.84, 1.42 mM PCF or 0.2 μM MG132 for 2 h or 1 h prior to UVB irradiation, respectively. # $P < 0.05$ , vs. Control; \* $P < 0.01$ , vs. UVB; \*\* $P < 0.01$ , 0.05 vs. UVB, UVB+PCF1; \*\*\* $P < 0.01$ , 0.05, 0.05 vs. UVB, UVB+ PCF1, UVB+ PCF2.





**Fig. 2.** Effect of PCF on UVB-induced the nuclear translocation of NF- $\kappa$ B/p65 in HaCaT cells. 1. Control; 2. Cells were irradiated with 20 mJ/cm<sup>2</sup> UVB; 3–4. Cells were pretreated with 1.42, 5.69 mM PCF for 2 h prior to UVB irradiation, respectively; The NF- $\kappa$ B localization in HaCaT cells exposed to UVB (20 mJ/cm<sup>2</sup>) was visualized with a fluorescence microscopy by indirect immunofluorescence using anti-NF- $\kappa$ B/p65 antibody.

oxygen species (ROS) generated by UVB are responsible for UVB-induced activation of NF- $\kappa$ B, it can promote the translocation of NF- $\kappa$ B [22]. In this study, we tried to confirm whether UVB activates NF- $\kappa$ B in HaCaT cells at the lower doses of UVB irradiation (20 mJ/cm<sup>2</sup>). As shown in Figs. 2 and 3, NF- $\kappa$ B was translocated to the nucleus in the response to UVB irradiation, which was most obvious at 3 h after UVB exposure in detection with immunofluorescence microscopy and Western blot analysis, respectively. Treatment with ROS scavenger NAC (5 mM) significantly inhibited the UVB induced NF- $\kappa$ B activation, as shown in Fig. 3. The results indicated that UVB radiation induced the activation of NF- $\kappa$ B and it may have been mediated by ROS.

#### 3.4. UVB radiation induced the phosphorylation and degradation of I $\kappa$ B $\alpha$

In most cell types, NF- $\kappa$ B is present in the cytoplasm in the form of a complex with its inhibitory protein I $\kappa$ B $\alpha$ , the complexes primarily exists in an inactive stage, and upon activation, I $\kappa$ B $\alpha$  undergoes a

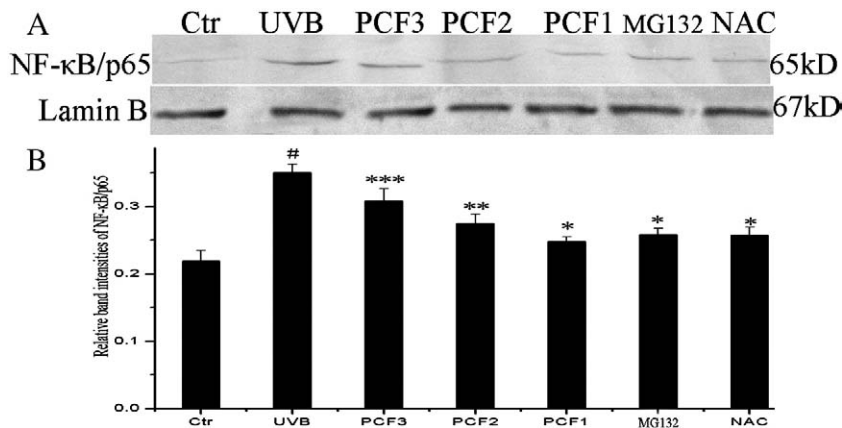
phosphorylative degradation through an ubiquitin-dependent pathway, which results in a translocation of NF- $\kappa$ B to nucleus [11]. The phosphorylative degradation, through the phosphorylation at serine-32 and 36 of I $\kappa$ B $\alpha$ , is a major mechanism of NF- $\kappa$ B activation [23]. And the I $\kappa$ B $\alpha$  degradation process is often activated by oxidants. So we evaluated whether UVB induced NF- $\kappa$ B activation through the phosphorylative degradation of I $\kappa$ B $\alpha$ . Firstly a kinetic analysis of I $\kappa$ B $\alpha$  phosphorylation and degradation were detected, as shown by the Western blot analysis (Fig. 4A), UVB exposure (20 mJ/cm<sup>2</sup>) to the HaCaT cells was found to result in an increased phosphorylative degradation of I $\kappa$ B $\alpha$  protein at 1 h, obviously at 3 h, and the phosphorylative degradation was decreased after 6 h, the expression at 24 h came to the baseline. Then, we assessed whether ROS is involved in this phosphorylative degradation process by using ROS scavenger NAC at 3 h after irradiation. As shown in Fig. 4B, the UVB-induced I $\kappa$ B $\alpha$  degradation was not observed when the cells were pretreated with NAC. Our results indicated that UVB-induced NF- $\kappa$ B activation in HaCaT cells is dependent on I $\kappa$ B $\alpha$  phosphorylation and degradation, which could be increased by ROS.

#### 3.5. UV radiation also decreased I $\kappa$ B mRNA expression so as to down-regulate the total amount of I $\kappa$ B

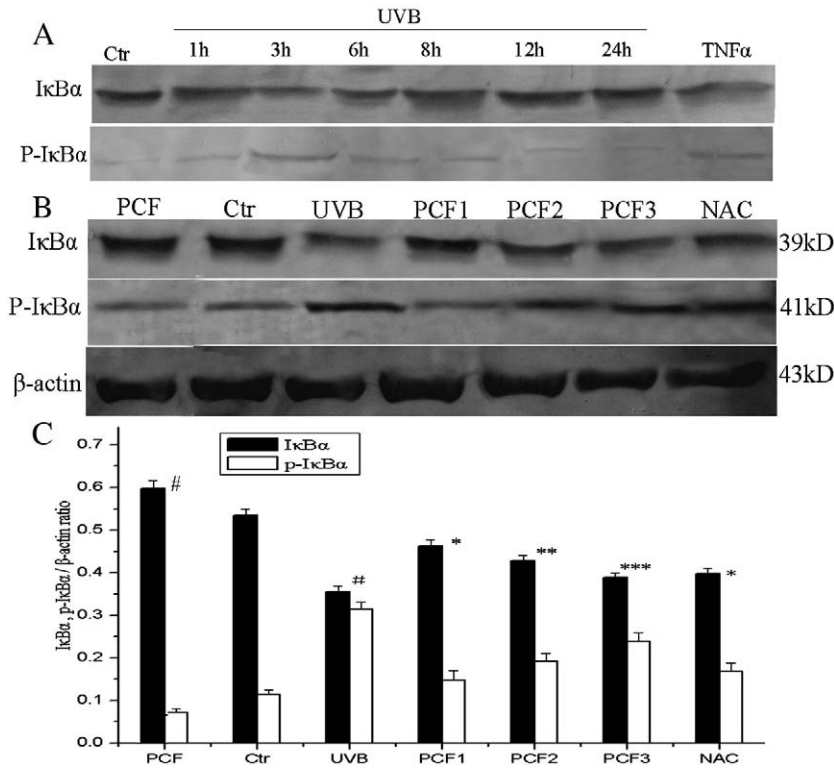
In order to investigate the possible mechanisms of UV radiation active NF- $\kappa$ B furtherly, we examined the mRNA level of I $\kappa$ B by using RT-PCR. The result showed that UV radiation decreased the I $\kappa$ B mRNA expression as shown in Fig. 5. Our study indicated that UV light down-regulates the total amount of I $\kappa$ B through decreasing I $\kappa$ B mRNA expression. Without a continuous supply of newly synthesized I $\kappa$ B, the existing I $\kappa$ B is degraded through a polyubiquitin-dependent proteasomal pathway leading to NF- $\kappa$ B activation.

#### 3.6. UVB radiation induced the activation of IKK $\alpha$

In fact, numerous extracellular stimuli can activate NF- $\kappa$ B through signaling pathways, which activate an IKK complex [24] that phosphorylates I $\kappa$ B on serine-32 and serine-36. The phosphorylation of I $\kappa$ B leads to its ubiquitination and ultimate degradation by the proteasome [11], allowing NF- $\kappa$ B to translocate to the nucleus where it activates the expression of genes. IKK is the key regulatory complex required for NF- $\kappa$ B activation of gene transcription [24]. As our studies have shown that UVB-mediated activation of NF- $\kappa$ B in HaCaT cells through a phosphorylative degradation of I $\kappa$ B $\alpha$ , we asked whether or not IKK plays a role in this process. As shown in Fig. 6, UVB exposure significantly increased the activation of IKK $\alpha$ . Our



**Fig. 3.** Effect of PCF, NAC and MG132 on UVB-induced NF- $\kappa$ B/p65 nuclear protein expression in HaCaT cells. A, Ctr—control; UVB— Cells were irradiated with 20 mJ/cm<sup>2</sup> UVB; PCF1–3, NAC — Cells were pretreated with 5.69, 2.84, 1.42 PCF, 5 mM NAC for 2 h prior to UVB irradiation respectively. MG132— Cells were pretreated with 0.2  $\mu$ M MG132 for 1 h prior to UVB irradiation. After irradiation, cells were incubated for 3 h, and NF- $\kappa$ B/p65 were 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 NF- $\kappa$ B/p65 over Lamin B. <sup>#</sup> $P$ <0.01, vs. control; <sup>\*</sup> $P$ <0.01, vs. UVB; <sup>\*\*</sup> $P$ <0.01, 0.05 vs. UVB, UVB+PCF1; <sup>\*\*\*</sup> $P$ <0.01, 0.05, 0.05 vs. UVB, UVB+PCF1, UVB+PCF2.

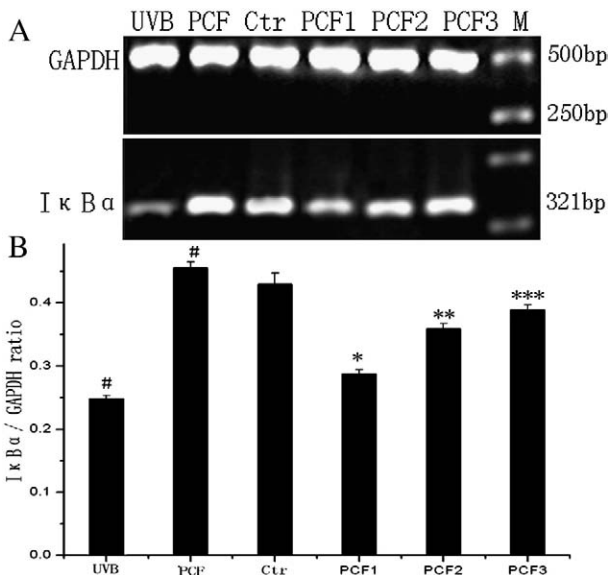


**Fig. 4.** Effect of PCF and NAC on UVB-induced IκBα and p-IκBα protein expression in HaCaT cells. A, Ctr—control; UVB— Cells were irradiated with 20 mJ/cm<sup>2</sup> UVB; TNFα—TNFα treated control in 3 h; after irradiation, cells were harvested at indicated time (1, 3, 6, 8, 12, 24 h), and IκBα and p-IκBα were detected by western blot. B, PCF— Cells were pretreated with 2.84 mM PCF for 2 h; PCF1–3, NAC — Cells were pretreated with 5.69, 2.84, 1.42 PCF, 5 mM NAC for 2 h prior to UVB irradiation respectively. After irradiation, cells were incubated for 3 h, and IκBα and p-IκBα were 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 IκBα and p-IκBα over β-actin. #*P*<0.01, vs. control; \**P*<0.05, vs. UVB; \*\**P*<0.01, 0.05 vs. UVB, UVB+PCF1; \*\*\**P*<0.01, 0.05, 0.05 vs. UVB, UVB+PCF1, UVB+PCF2.

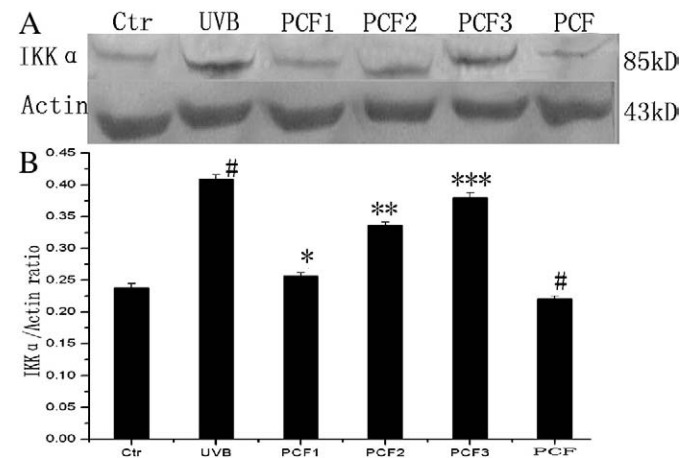
results demonstrated that UVB-radiation-induced the activation of IKKα/IκBα/NF-κB signaling pathway played an important role in UVB-induced apoptosis of HaCaT cells.

**3.7. PCF inhibited UVB-induced activation of NF-κB through inhibition of decreasing and activation of IκBα and IKKα, respectively**

As we have shown that UVB-induced activation of NF-κB increased the apoptosis in UVB-induced HaCaT cells, and PCF could effectively inhibit HaCaT cells apoptosis after UVB exposure or non-exposure, so



**Fig. 5.** Effect of PCF on UVB-induced IκBα mRNA expression in HaCaT cells. Ctr—control; UVB— Cells were irradiated with 20 mJ/cm<sup>2</sup>UVB; PCF— Cells were pretreated with 2.84 mM PCF for 2 h; PCF1–3 — Cells were pretreated with 5.69, 2.84, 1.42 PCF for 2 h prior to UVB irradiation respectively. After irradiation, cells were incubated for 3 h, and IκBα 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 IκBα over GAPDH. #*P*<0.01, vs. control; \**P*<0.05, vs. UVB; \*\**P*<0.01, 0.05 vs. UVB, UVB+PCF1; \*\*\**P*<0.01, 0.05, 0.05 vs. UVB, UVB+PCF1, UVB+PCF2.



**Fig. 6.** Effect of PCF on UVB-induced IKKα protein expression in cytosol in HaCaT cells. Ctr—control; UVB— Cells were irradiated with 20 mJ/cm<sup>2</sup> UVB; PCF— Cells were pretreated with 2.84 mM PCF for 2 h; PCF1–3— Cells were pretreated with 5.69, 2.84, 1.42 PCF for 2 h prior to UVB irradiation respectively. After irradiation, the postnuclear lysates were prepared at 3 h, and IKKα were 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 IKKα over β-actin. #*P*<0.05, vs. control; \**P*<0.05, vs. UVB; \*\**P*<0.05, 0.05 vs. UVB, UVB+PCF1; \*\*\**P*<0.05, 0.05, 0.05 vs. UVB, UVB+PCF1, UVB+PCF2.

we investigate whether the anti-apoptotic effect of PCF made by its ability to modulate the NF- $\kappa$ B signaling pathway. Therefore, we determined whether PCF could inhibit activation of the transcription factor NF- $\kappa$ B/p65, which plays an important role in UVB-induced apoptosis of HaCaT cells.

As shown in Figs. 2 and 3, pretreatment of HaCaT cells with 5.69, 2.84 or 1.42 mM PCF before UVB irradiation markedly abrogated the UVB-induced activation and subsequently translocation of NF- $\kappa$ B to the nucleus in a dose-dependent manner.

UVB exposure to HaCaT cells resulted in the phosphorylation and degradation of I $\kappa$ B $\alpha$  protein, and because of this degradation NF- $\kappa$ B/p65 is activated and translocated into the nucleus (Figs. 2, 3). We were interested to see whether the phosphorylation and degradation of I $\kappa$ B $\alpha$  is inhibited by PCF, which will in turn inhibit the activation and translocation of NF- $\kappa$ B/p65. We observed that pretreatment with 2.84 mM PCF only could decrease the phosphorylation and degradation of I $\kappa$ B $\alpha$  protein in un-irradiated cells and preincubated with different concentrations of PCF drastically decreased UVB-induced phosphorylation and degradation of I $\kappa$ B $\alpha$  protein in a dose-dependent manner as evident by Western blot analysis and the relative density of the bands (Fig. 4B). As we have shown that UV radiation decreased the I $\kappa$ B mRNA expression, so we asked whether PCF could influence the mRNA expression of I $\kappa$ B $\alpha$ . We found that Supplementation of PCF to HaCaT cells prior to UVB exposure or non-exposure markedly increased I $\kappa$ B $\alpha$  mRNA expression in a dose-dependent manner in UVB-exposed HaCaT cells (Fig. 5).

The induction of IKK $\alpha$  activity has been shown to be necessary for I $\kappa$ B $\alpha$  protein phosphorylation/degradation induced by UVB radiation. To evaluate the possible inhibitory mechanism of PCF on I $\kappa$ B $\alpha$  protein degradation, we measured IKK $\alpha$  protein level in cytosol. Our Western blot analysis indicated that pretreatment of HaCaT cells with PCF significantly inhibit UVB-induced activation of IKK $\alpha$  in a concentration-dependent manner (Fig. 6).

Our results demonstrated that PCF effectively inhibited UVB-induced NF- $\kappa$ B activation and translocation to the nucleus though blocking I $\kappa$ B $\alpha$  phosphorylation/degradation and mRNA expression in UVB-exposed HaCaT cells, which mediated by inhibition of activation of IKK $\alpha$  in a concentration-dependent manner.

#### 4. Discussion

Ultraviolet irradiation (UVR) is one of the harmful environmental factors that cause skin photoaging, immunosuppression and photocarcinogenesis. UVR induces the generation of reactive oxygen species (ROS), which are responsible for the photooxidative damage on nucleic acids, lipids and proteins and also initiate cellular damage and apoptosis [25]. Because of the increasing awareness of the harmful effects of UV radiation, there is a general need for safe and effective materials. Our previous report demonstrated that PCF could inhibit production of ROS and have potent antioxidant activity [19], but its anti-apoptotic mechanisms were not clearly understood. In this study, we investigated the effects of PCF on UVB-induced apoptosis and the signaling pathways in HaCaT cells. We conclude that PCF could attenuate HaCaT cells apoptosis and modulated NF- $\kappa$ B signaling pathway in HaCaT cells.

Apoptosis is a tightly regulated form of cell death and a multifactor-related process, including gene expression and mutation. The execution of the apoptosis program is characterized by morphological and biochemical changes. NAC as a scavenging of ROS, protecting Jurkat cells against apoptosis produced by UVC irradiation [26]. This study observed its protective effects on UVB induced apoptosis in HaCaT cells. Our Hoechst 33258 stain demonstrated that UV irradiation obviously induced the apoptosis; PCF and NAC protected the cells from apoptosis. The results suggested that ROS was involved in UVB-induced HaCaT cells apoptosis, which could be effectively inhibited by PCF in a concentration-dependent manner.

It has been reported that UVB-induced skin photoaging processes are ascribable to the activation of a transcription factor, nuclear factor  $\kappa$ B (NF- $\kappa$ B). A number of studies have confirmed that oxidative stress induced by increased generation of ROS is involved in the activation of NF- $\kappa$ B [27]. So it was of interest to examine the protective effect of PCF on UVB induced apoptosis through NF- $\kappa$ B signaling pathway. In this study we observed NF- $\kappa$ B activation in HaCaT cells at a UVB dose of 20 mJ/cm<sup>2</sup>. The role of NF- $\kappa$ B activation in UV-induced apoptosis is controversial, appearing to be dependent upon the type of cells and stimuli. In many cell types, the induction of the NF- $\kappa$ B signaling cascade serves to protect cells from a variety of cellular stresses [28]. In this study, we showed that MG132 effectively prevented UVB-induced apoptosis, suggesting that in our system, activation of NF- $\kappa$ B resulted in apoptosis. Therefore, we were interested to see the possible mechanisms of UVB induced NF- $\kappa$ B activation which caused HaCaT cells apoptosis.

NF- $\kappa$ B complexes are predominantly cytoplasm and thus transcriptionally inactive until a cell is activated by a relevant stimulus such as UV. The activation of NF- $\kappa$ B by the extracellular inducers depends on the phosphorylation and subsequent degradation of I $\kappa$ B proteins which is achieved through the action of a family of serine/threonine kinases known as IKK [29]. The IKK complex is believed to be an important site for integrating signals that regulate the NF- $\kappa$ B pathway [30]. Therefore, the inhibitors of IKK have long been sought as specific regulators of NF- $\kappa$ B. In our present study, we have demonstrated that NF- $\kappa$ B is activated and translocated to the nucleus in HaCaT cells upon exposure to UVB radiation when measured by Western blot analysis and immunofluorescence staining. UVB exposure also resulted in an increased phosphorylation and degradation of I $\kappa$ B $\alpha$  protein and decreased I $\kappa$ B mRNA expression. Additionally, our data also demonstrated that UVB radiation upregulated the levels of IKK. These data suggest that UVB radiation induced activation of NF- $\kappa$ B dependent on inhibition of degradation and activation of I $\kappa$ B $\alpha$  and IKK $\alpha$  respectively, which play an important role in apoptosis.

Our previous studies have demonstrated that in vitro treatment of HaCaT cells with PCF inhibited UVA-induced apoptosis through its effects on inhibiting UVA-induced phosphorylation of MAPK proteins [31]. Therefore, we determined whether PCF could inhibit activation of the transcription factor NF- $\kappa$ B/p65, which is a downstream target of the MAPK signal transduction pathways. In our present study, we demonstrated that PCF effectively inhibited UVB-induced NF- $\kappa$ B/p65 activation and translocation to the nucleus in a concentration-dependent manner. PCF also blocked I $\kappa$ B $\alpha$  phosphorylation and degradation while increased its mRNA expression in UVB-exposed HaCaT cells in a concentration-dependent manner. Our data suggests that the inhibitory effect of PCF on NF- $\kappa$ B/p65 activation may be mediated through the inhibition of proteolysis of I $\kappa$ B $\alpha$  protein. It is well documented that through a protein–protein interaction, I $\kappa$ B $\alpha$  is bound to NF- $\kappa$ B/p65 and thus prevents migration of NF- $\kappa$ B/p65 into the nucleus [32]. Furthermore, we observed that PCF markedly inhibited IKK $\alpha$  protein expression in a concentration-dependent manner. These data indicated that in vitro treatment of HaCaT cells with PCF inhibited UVB-induced activation and nuclear translocation of NF- $\kappa$ B/p65 through the inhibition of activation of IKK $\alpha$  and degradation of I $\kappa$ B $\alpha$  proteins so as to protect UVB-induced apoptosis.

Saliou et al. [21] reported that agents that scavenge ROS inhibit NF- $\kappa$ B activation, suggesting the key role of ROS in NF- $\kappa$ B pathway. In this study, pretreatment with NAC also inhibited UVB-induced NF- $\kappa$ B activation and I $\kappa$ B $\alpha$  phosphorylation/degradation. Therefore, the inhibitory effect of PCF on NF- $\kappa$ B pathway may partly depend on its property as a ROS scavenger.

In summary, our observations suggest that UVB irradiation induced NF- $\kappa$ B activation though IKK $\alpha$ /I $\kappa$ B $\alpha$ /NF- $\kappa$ B signaling pathway which increased the sensitivity of apoptosis in HaCaT cells. Thus, ROS and NF- $\kappa$ B pathway were involved in the inhibitory effect of PCF on UVB-induced apoptosis of HaCaT cells. Furthermore, PCF partly though

decreasing intracellular ROS level and modulating the NF- $\kappa$ B signaling pathway to exert its protective effect. PCF 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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