Free Radical Research, January 2008; 42(1): 12–19

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Involvement of ROS/ASMase/JNK signalling pathway in inhibiting UVA-induced apoptosis of HaCaT cells by polypeptide from *Chlamys farreri*

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Accepted by Dr. J. Keller

(Received 14 September 2007; in revised form 20 October 2007)

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Abstract

Polypeptide from *Chlamys farreri* (PCF), a novel marine active material isolated from gonochoric Chinese scallop *C. farreri*, has potential antioxidant activity and protective effect against ultraviolet (UV) irradiation. The aim was to investigate whether PCF protects HaCaT cells from apoptosis induced by UVA and explore related molecular mechanisms. The results showed that PCF significantly prevented UVA-induced apoptosis of HaCaT cells. PCF not only strongly reduced the intracellular reactive oxygen species (ROS) production, but also diminished expression of acid sphingomyelinase (ASMase) and phosphorylated JNK in HaCaT cells radiated by UVA in a dose-dependent manner. Pre-treatment with ROS scavenger NAC, ASMase inhibitor Desipramine or JNK inhibitor SP600125 was found to effectively prohibit UVA-induced apoptosis and Desipramine markedly blocked phosphorylation of JNK. So it is concluded that PCF obviously protects HaCaT cells from apoptosis induced by UVA and protective effects may attribute to decreasing intracellular ROS level and blocking ASMase/JNK apoptotic signalling pathway.

Keywords: *Polypeptide from Chlamys farreri, ultraviolet A, apoptosis, reactive oxygen species, acid sphingomyelinase, JNK*

Introduction

It has been well elucidated that ultraviolet (UV) irradiation has many deleterious effects on human skin, including sunburn, premature ageing and skin cancer [1]. The solar radiation that can reach the surface of the earth consists of 90–99% UVA (320–400 nm) and 1–10% UVB (280–320 nm). Although UVC (200–280 nm) damages skin to a larger extent than UVA or UVB, it is almost all absorbed by the ozone layer [2,3]. UVA not only penetrates more deeply into the skin than UVB, reaching the epidermis and the dermis, but also destroys collagen, elastic fibres and fibroblast. In addition, the majority of UVA directly gets to the ground without being absorbed by the atmosphere

A number of studies have confirmed that UV significantly contributes to apoptosis of human keratinocytes *in vivo* and *in vitro* [4]. UV-mediated apoptosis is a highly complex event to a large extent unknown. In order to diminish the damage of skin from UV, many protective agents involving vitamins C, E and β-carotene have gained considerable attention over the past decade. Polypeptide from *Chlamys farreri* (PCF), Mr = 879 kDa, is a novel marine active material isolated from gonochoric Chinese scallop

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2008 Informa UK Ltd.
DOI: 10.1080/10715760701762415

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C. farreri applying modern marine life engineering. As a water-soluble octapeptide, PCF consists of Pro, Asn, Ser, Thr, Arg, Hyl, Cys and Gly (Appl.: 00111426.9, China). Previous studies from our laboratory have indicated that PCF has potential antioxidant activity and protective effect against UVA irradiation in hairless mice skin and human skin fibroblast [5,6], but its anti-apoptotic mechanisms were not understood.

Reactive oxygen species (ROS) play a very important role in apoptosis induction under both physiological and pathological conditions. UV irradiation can induce ROS accumulation followed by apoptosis [7]. Ceramide is an important second messenger and its generation may involve hydrolysis of sphingomyelin (SM) by various sphingomyelinases (SMases). These SMases are defined by different PH optima as acid sphingomyelinase (ASMase), neutral sphingomyelinase (NSMase) or alkaline sphingomyelinase [8]. ASMase is involved in a stress-induced signalling pathway in many types of cells [9]. The c-Jun NH_2-terminal kinase (JNK) is known as stress-activated protein kinase (SAPK), which belongs to the mitogen-activated protein kinase (MAPK) superfamily [10,11]. The JNK signalling pathway is activated by various cellular stimuli such as UV light [12], radiation [13], ceramide [14] and TNF-α [15]. Recent evidence has shown that the mechanism of apoptosis induced by UV may involve ASMase [16,17] and JNK [18]. Many articles also indicated that ASMase plays an obligatory part in UV-induced apoptosis and JNK is downstream kinase of the SMase signalling pathway [19,20]. So it was of interest to examine the protective effect of PCF on UVA-induced apoptosis through the ASMase/JNK pathway.

Based on the important roles of ROS, ASMase and JNK in UV-induced apoptosis, in the present study we investigated the possibility that PCF inhibited UVA-induced apoptosis in HaCaT cells through ROS/ASMase/JNK apoptotic signalling pathway.

Materials and methods

Materials

PCF was purified (purity $>$ 96%) and analysed by HPLC (Yellow Sea Fishery Research Institute, China), dissolved in sterile deionized water, stored at 4°C. ASMase inhibitor Desipramine, JNK inhibitor SP600125 and ROS scavenger NAC were purchased from Sigma. Antibodies against JNK and ASMase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-JNK antibody was purchased from Cell Signaling Technology™ (Beverly, MA). Anti-β-actin antibody and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were acquired from Beyotime Biotechnology (China). The primers of ASMase and GAPDH were designed and synthetized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China). All the other chemicals used were of the highest grade commercially available.

Cell culture and UVA treatment

The human keratinocyte cell line HaCaT, kindly provided by Dr Bo-Xiao Ding (Yonsei University, Korea), was grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were randomly divided into several groups including control group (normal cultural cells), model group (UVA-irradiated cells), PCF group (PCF pre-treated and then UVA-irradiated cells) and the inhibitor group (the corresponding inhibitor pre-treated and then UVA-irradiated cells). When cells were grown to 80–90% confluence, UVA exposure was performed with UVA lamps (Beijing Normal University, China) and took 2.5 h to reach the fluence 8 J/cm^2. PCF, NAC, Desipramine and SP600125 were added in medium 2, 2, 2 and 0.5 h, respectively, before irradiation. During irradiation, medium was discarded and cells were placed in PBS. After irradiation, medium was exchanged and cells were cultured normally.

Hoechst 33258 staining

At 18 h after UVA 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 500 cells were counted. Apoptotic cell death was then calculated as a percentage of apoptotic cells over the total blue fluorescent protein-positive cells.

DNA fragmentation assay

A DNA fragmentation assay was carried out as reported by others. Briefly, cells were washed with PBS and harvested at 18 h after UVA 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

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in TE buffer and incubated with RNase A (20 μg/l) at 37°C for 30 min. DNA fragmentation was analysed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light.

Measurement of ROS production

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) in multi-functional microplate reader (Tecan Safire 2, Switzerland). Cells were harvested immediately after UVA radiation and adjusted to 1×10^7/ml, then incubated with 10 μmol/L DCFH-DA at 37°C for 30 min. Cells were subsequently washed twice with D-Hanks and collected. Fluorescence intensity of 100 μl cell suspension was detected by multi-functional microplate reader at an excitation wavelength of 480 nm and at an emission wavelength of 530 nm.

Detection of ASMase by RT-PCR

At 1 h [19] after UVA treatment, total RNA was extracted according to the manufacturer's protocol with Trizol reagent (Takara, China). Then total RNA was reverse transcribed following the manufacturer's instructions (Exscript™ RT reagent kit, Takara, China). The total volume of PCR reaction was 50 μl according to PCR Amplification kit (Takara, China). The PCR conditions were at 95°C for 3 min, followed by 35 cycles at 94°C for 40 s, at 60°C for 40 s and at 72°C for 40s. The final extension step was at 72°C for 3 min. PCR products were separated by 1.5% agarose gel electrophoresis and photographed. Sizes of PCR products were ASMase, 161bp; GAPDH, 509bp. The sequence of PCR primers used in this study were: ASMase, forward primer, 5′-GCC CAA TCT GCA AAG GTC TAT TC-3′; reverse primer, 5′-CAT GTC ATC CTC AAA GAG GTG GA-3′; and GAPDH, forward primer, 5′-CGT GGA AGG ACT CAT GAC CA-3′; reverse primer, 5′-TCC AGG GGT CTT ACT CCT TG-3′. Purification and gene sequencing of the PCR product were accomplished by Shanghai Sangon Biological Engineering Technology & Services Co. The intensity of each RT-PCR band was analysed by Quantity One software.

Western blot analysis for ASMase and JNK

ASMase, JNK and phosphorylated JNK (P-JNK) were analysed by western blot. At 1 h (for ASMase) [19] or 6 h (for P-JNK and JNK) [21] after UVA treatment, 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); 40 μg of protein 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 ASMase (1:200), P-JNK (1:1000), JNK (1:200), or 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 ECL detection kit (Santa Cruz

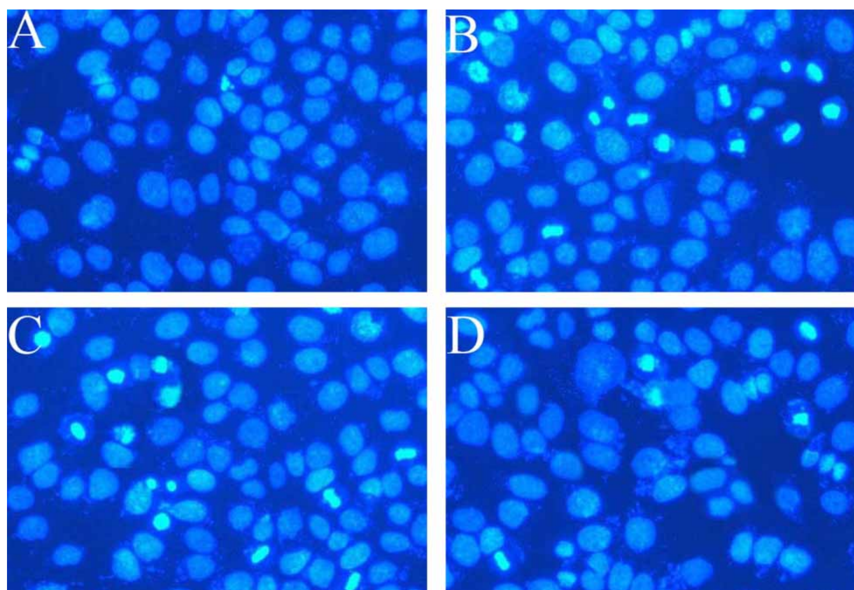

Figure 1. Effect of PCF on UVA-induced apoptosis in HaCaT cells by Hoechst 33258 staining. (A) control; (B) UVA model; (C) and (D) HaCaT cells were pre-treated with 1.42 or 5.68 mM PCF for 2 h prior to UVA irradiation, respectively.

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Table I. Effect of PCF on apoptosis in UVA-irradiated HaCaT cells ($\bar{x} \pm s$, $n = 3$).

Groups	Apoptotic cells (%)
Control	0.87 ± 0.31
UVA model	26.47 ± 2.81[a]
UVA+PCF1 (1.42 mM)	17.13 ± 2.21[b]
UVA+PCF2 (2.84 mM)	12.2 ± 1.51[bc]
UVA+PCF3 (5.68 mM)	6.57 ± 2.08[bcd]

Indicated are mean and SD of three independent experiments. [a]$p < 0.01$ vs control; [b]$p < 0.01$ vs UVA; [c]$P < 0.01$ vs UVA+PCF1; [d]$p < 0.01$ vs UVA+PCF2.

Biotechnology, Inc, USA). The densities of sample bands were analysed with Quantity One analysis software.

Statistical analysis

All data were presented as the mean ± SD. Each experiment was repeated at least three times to confirm the reproducibility of the findings. Multiple groups were analysed by one-way analysis of variance (ANOVA) followed by a post-hoc Student Newman-Keuls' test. $p < 0.05$ was considered statistically significant.

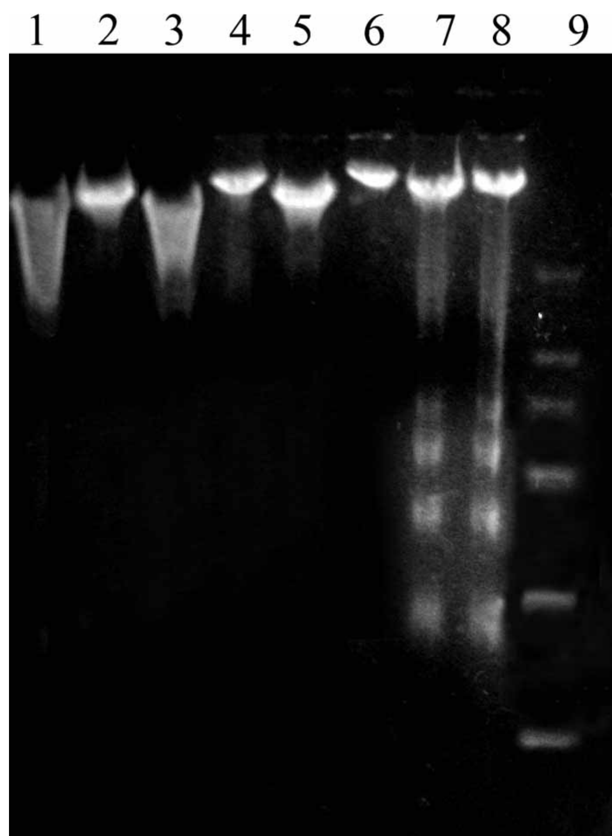

Figure 2. Effect of PCF, Desipramine, SP600125 and NAC on UVA induced-DNA fragmentation in HaCaT cells. Lane 1, control; lanes 2–3, cells were pre-treated with 25 μmol/L Desipramine for 2 h or 20 μmol/L SP600125 for 0.5 h prior to UVA irradiation, respectively; lanes 4–7, cells were pre-treated with 5 mM NAC, 5.68, 2.84 or 1.42 mM PCF for 2 h prior to UVA irradiation, respectively; lane 8, cells were irradiated with 8 J/cm^2 UVA; lane 9, standard base pair marker (2000, 1000, 750, 500, 250, 100 bp).

Results and discussion

Inhibitory effect of PCF on apoptosis in UVA-irradiated HaCaT cells

First, we successfully established the UVA-induced apoptosis model of HaCaT cells and then we conducted Hoechst 33258 staining for the identification of apoptotic nuclei. As shown in Figure 1, in model group (Figure 1B), cells showed condensed bright nuclei typical of apoptotic dead cells, while almost no apoptotic nuclei were observed in cells of control (Figure 1A). When cells were pre-treated with 1.42 mM PCF (Figure 1C) and 5.68 mM PCF (Figue 1D), the number of apoptotic cells was concentration-dependently decreased. Our data from Table I also support the view of the protective effect of PCF on UVA-induced apoptosis in a dose-dependent manner.

DNA ladder is another marker of cell apoptosis, so DNA fragmentation assay was examined. At 18 h after irradiation, an obvious DNA ladder appeared (Figure 2, lane 8) in comparison with control group (Figure 2, lane 1). Pre-treatment with 5.68, 2.84 or 1.42 mM PCF for 2 h prior to irradiation markedly attenuated UVA-induced DNA fragmentation (Figure 2, lanes 5–7). The above-mentioned results indicate that PCF effectively inhibits apoptosis of HaCaT cells after UVA exposure.

Effect of PCF on intracellular ROS level in UVA-treated HaCaT cells

UV irradiation is a potent inducer of various reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide (H_2O_2) and hydroxyl radicals. After UVA exposure, ROS are generated and lead to apoptosis of cells [7,22]. NAC is a cell permeable ROS scavenger and could completely block ROS generation [23]. This study observed the protective effects of NAC on UVA induced apoptosis in HaCaT

Table II. Effect of PCF on ROS levels in UVA-irradiated HaCaT cells ($\bar{x} \pm s$, $n = 3$).

Groups	ROS (RFU)
Control	38265.33 ± 2301.95
UVA model	46835.00 ± 1692.62[a]
UVA+PCF1 (1.42 mM)	43473.00 ± 741.26[bce]
UVA+PCF2 (2.84 mM)	40041.33 ± 1310.96[bd]
UVA+PCF3 (5.68 mM)	36643.00 ± 1431.40[b]

Cells were harvested immediately after irradiation and ROS levels were detected in multi-functional microplate reader. Indicated are mean and SD of three independent experiments. [a]$p < 0.01$ vs control; [b]$p < 0.05$ vs UVA; [c]$p < 0.01$, [d]$p < 0.05$ vs UVA+PCF3; [e]$p < 0.05$ vs UVA+PCF2.

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cells (Figure 2, lane 4), suggesting that UVA-induced apoptosis in HaCaT cells is mediated by ROS.

As shown in Table II, UVA irradiation significantly increased the intracellular level of ROS. Pre-treatment with PCF significantly inhibited the elevated intracellular concentration of ROS in a dose-dependent manner. UVA-induced ROS production can be efficiently attenuated by PCF, indicating that impairment of ROS formation is responsible for the anti-apoptotic effect of PCF.

Effect of PCF on expression of ASMase induced by UVA irradiation in HaCaT cells

To provide further evidence for the role of ASMase in UVA initiated cell death, we performed inhibition experiments. With the ability to enhance degradation of zymoprotein, Desipramine has been identified as an inhibitor of ASMase. In this study, we showed that Desipramine effectively abolished UVA-induced apoptosis (Figure 2, lane 2), suggesting that, in our system, ASMase plays an important role in mediating the apoptotic effect of UVA irradiation. Deficiency in ASMase has been shown to diminish the response of endothelial and neuronal cells to radiation-induced apoptosis [24].

It has been reported that 50 J/m^2 UVC maximally increased ASMase expression on the outer leaflet of the plasma membrane within 1 min [16]. Langmann et al. [25] have suggested that the activity of ASMase is mainly determined by its expression. As we assumed a critical role of ASMase in UVA-induced apoptosis, we investigated the expression of this enzyme. We treated HaCaT cells with different concentrations of PCF before UVA exposure and then tested for ASMase by the method of RT-PCR. As shown in Figure 3, lane 5, UVA irradiation strongly stimulated the expression of ASMase, suggesting that ASMase contributes to the induction of the apoptotic cascade in HaCaT cells. When cells were pre-incubated with PCF for 2 h, the level of ASMase-mRNA was concentration-dependently decreased (Figure 3, lanes 1–3). Furthermore, sequence analysis of the 161 bp product confirmed the amplification of the respective ASMase fragment (Figure 4). The result coincided with DNA sequence of ASMase (GenBank accession no. X63600).

We also examined the expression of ASMase protein by western blot analysis for the further identification. As shown in Figure 5, lane 1, the level of ASMase increased significantly in UVA-treated HaCaT cells compared with untreated cells (Figure 5, lane 5). When cells were pre-incubated with different concentrations of PCF, expression levels decreased in a dose-dependent manner (Figure 5, lanes 2–4). So we inferred that PCF inhibited UVA-induced apoptosis through its effects on ASMase.

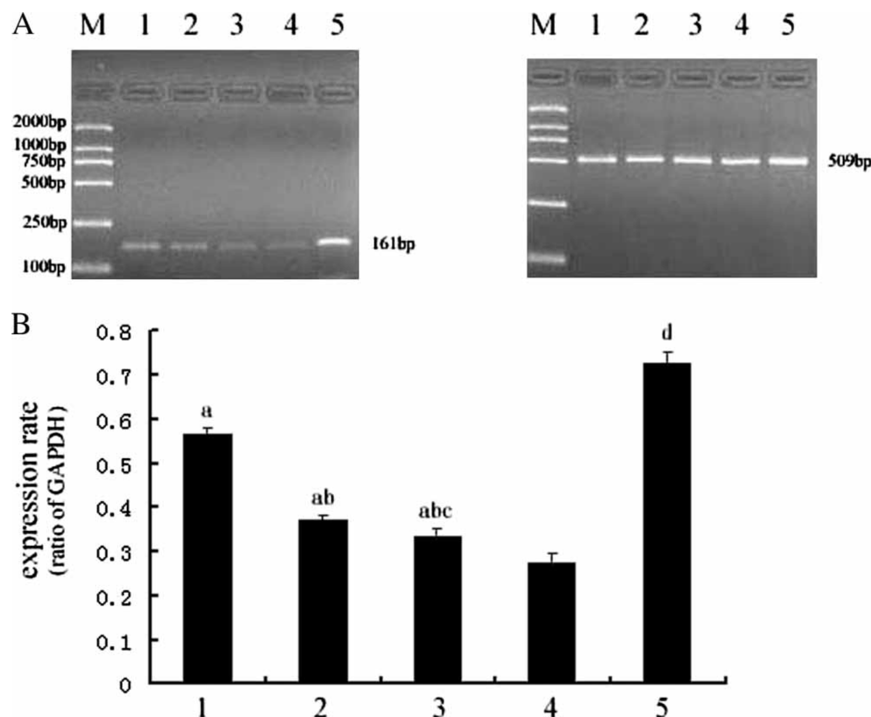

Figure 3. Effect of PCF on intracellular ASMase-mRNA level in UVA-radiated HaCaT cells. (A) lane M marker; lanes 1–3, cells were pre-treated with 1.42, 2.84 or 5.68 mM PCF for 2 h prior to UVA irradiation, respectively; lane 4, control; lane 5, cells were irradiated with 8 J/cm^2 UVA. Cells were harvested 1 h after irradiation and level of ASMase-mRNA 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 ASMase over GAPDH. $^a p<0.01$ compared with UVA irradiation group, $^b p<0.01$ compared with 1.42 mM PCF+UVA group, $^c p<0.05$ compared with 2.84 mM PCF+UVA group, $^d p<0.01$ compared with control.

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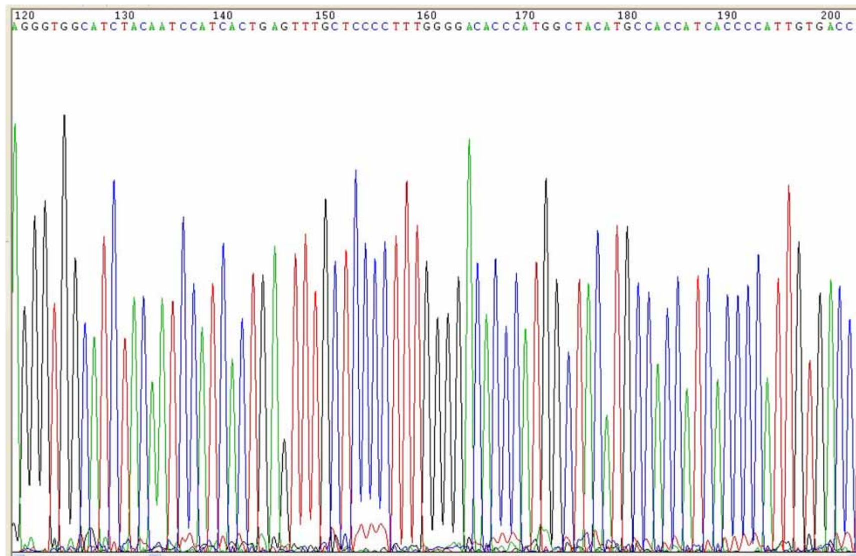

Figure 4. Sequence analysis graph of the PCR product.

Effects of PCF and Desipramine on activation of JNK induced by UVA

Many studies suggested that the JNK signal pathway plays a pivotal role in apoptosis of epithelial cell induced by UV [26]. There are three isoforms of JNK referred to JNK1, JNK2 and JNK3. JNK1 (Mr = 46 kDa) and JNK2 (Mr = 55 kDa) genes are ubiquitously expressed, but expression of JNK3 gene is restricted to the brain and heart. The activation of JNK results in phosphorylation of multiple transcription factors such as c-Jun, ATF-2 and CHOP/GADD153 [8], then activation of pro-apoptotic genes and eventually apoptosis [27,28]. In this study we observed that JNK inhibitor SP600125 inhibited UVA-induced DNA fragmentation (Figure 2, lane 3),

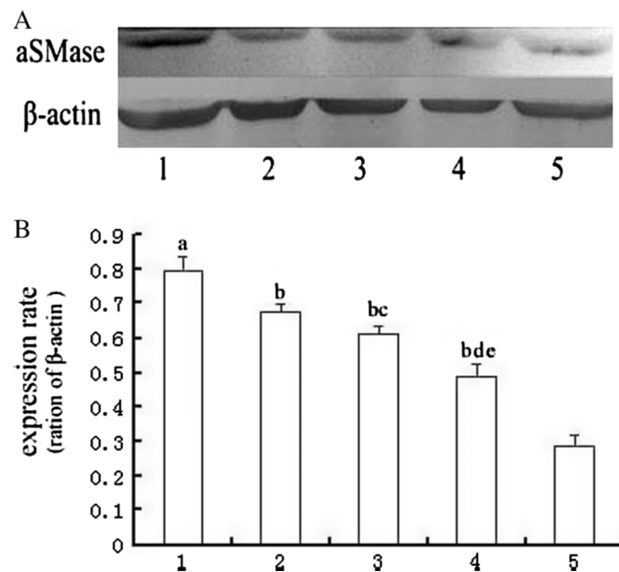

Figure 5. Effect of PCF on UVA-induced ASMase protein level in HaCaT cells. (A) Lane 1, cells were irradiated with 8 J/cm^2 UVA; lanes 2–4, cells were pre-treated with 1.42, 2.84, 5.68 mM PCF for 2 h prior to UVA irradiation, respectively. After irradiation, cells were incubated for 1 h and ASMases were detected by western blot; lane 5, control. Results shown are representative of three independent experiments. (B) Quantification of (A) results. Results were expressed as the ratio of expression level of ASMase over β-actin. $^{a}p < 0.01$ compared with control, $^{b}p < 0.01$ compared with UVA irradiation group, $^{c}p < 0.05$, $^{d}p < 0.01$ compared with 1.42 mM PCF+UVA group, $^{e}p < 0.01$ compared with 2.84 mM PCF+UVA group.

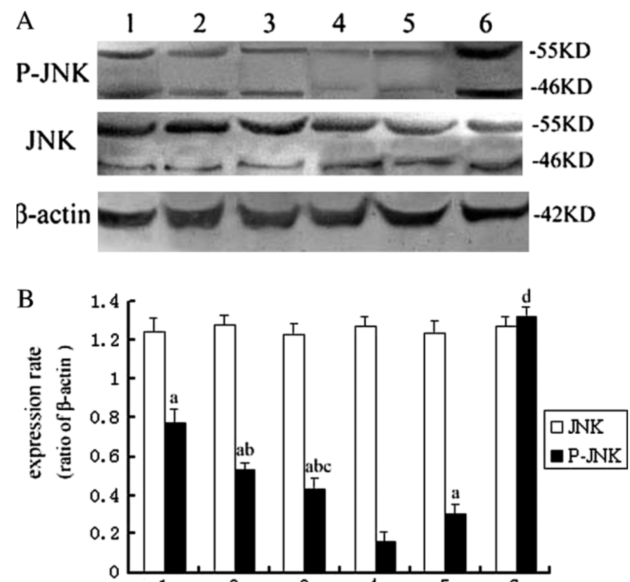

Figure 6. Effect of PCF and Desipramine on UVA-induced JNK activation in HaCaT cells. (A) Lanes 1–3, cells were pre-treated with 1.42, 2.84 or 5.68 mM PCF for 2 h prior to UVA irradiation, respectively; lane 4, control; lane 5, cells were pre-treated with 25 μmol/L Desipramine for 2 h prior to UVA irradiation; lane 6, cells were irradiated with 8 J/cm^2 UVA. Cells were harvested 6 h after irradiation and JNK activation 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 JNK or total JNK over β-actin. $^{a}p < 0.01$ compared with UVA irradiation group, $^{b}p < 0.01$ compared with 1.42 mM PCF+UVA group, $^{c}p < 0.05$ compared with 2.84 mM PCF+UVA group, $^{d}p < 0.01$ compared with control.

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suggesting that UVA-induced apoptosis in HaCaT cells is related to JNK.

In our experiments, UVA irradiation strongly stimulated the expression of phosphorylated JNK (Figure 6, lane 6), which is the active form of the kinase, while total levels of JNK were not affected. When cells were pre-incubated with PCF for 2 h, the levels of phosphorylated JNK were concentration-dependently decreased (Figure 6, lanes 1–3). These data provided evidence that PCF inhibits UVA-induced apoptosis through suppression of JNK activation.

Huang et al. [29] reported that UVB irradiation markedly induced JNK activation in normal human lymphoblast JY, while only very weak JNK activation was found in MS1418, which is a SMase-deficient lymphoblast cell line. In line with their report, we presented here strong evidence that ASMase and its products play an essential role in UV-induced JNK activation. Further studies showed that, when Desipramine, an inhibitor of ASMase, was used, the activation of JNK in cells damaged by UVA irradiation was blocked to a large extent (Figure 6, lane 5). So we confirmed that UVA-induced apoptosis occurs primarily through activation of JNK via the ASMase pathway in HaCaT cells and ASMase is on the top of the JNK signalling pathway. Furthermore, PCF could protect HaCaT cells from damage by UVA irradiation through inhibition of ASMase/JNK pathway.

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Conclusions

In summary, our observations suggest that ROS, ASMase and JNK are involved in the apoptosis of HaCaT cells induced by UVA. PCF may protect HaCaT cells from damage by UVA irradiation via decreasing intracellular ROS level and blocking ASMase/JNK signal pathway. PCF is a potential antioxidant and an effective protective agent against UVA-induced apoptosis in the human keratinocyte cell line HaCaT. Therefore, we believe that topical application of PCF helps prevent skin ageing and protect the natural barrier function of the skin.

Acknowledgements

The work was supported by the National Science Natural Foundation of China (No. 30471458) and Science Foundation of Shandong province (No. Y2003c02).

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