

## Original Article

# Polypeptide from *Chlamys farreri* restores endoplasmic reticulum (ER) redox homeostasis, suppresses ER stress, and inhibits ER stress-induced apoptosis in ultraviolet B-irradiated HaCaT cells

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**Abstract:** Objective: To investigate the effects of polypeptide from *Chlamys farreri* (PCF) on ultraviolet B (UVB)-induced apoptosis in human keratinocyte HaCaT cells. Methods: In HaCaT cells at 4 h or 18 h after UVB irradiation, the cell viability was measured by MTT assay. Cellular apoptosis was detected with annexin V-FITC/PI staining by flow cytometry. The expression levels of PDI, Ero-1 $\alpha$ , GRP78, and CHOP were assessed by Western blot analysis. Mitochondrial membrane potential (MMP) was measured by fluorescent probe JC-1. Caspase activities were detected with fluorogenic substrates. Results: PCF alleviated cell viability loss and inhibited apoptosis in HaCaT cells after UVB irradiation. Moreover, PCF increased the expression levels of PDI and Ero-1 $\alpha$ , which were related with the ER redox homeostasis. Furthermore, PCF treatment inhibited the expression of GRP78 at 4 h after UVB irradiation, and suppressed CHOP expression at 18 h post-irradiation, indicating that PCF could inhibit UVB-evoked ER stress in the early stage post-irradiation, and suppress the ER stress-induced apoptosis in the late stage. In addition, PCF alleviated UVB-induced MMP loss, and inhibited the activation of caspase-9/-3, in HaCaT cells after UVB irradiation. On the other hand, MMP loss and caspase-9/-3 activation could be partly blocked by the ER stress inhibitor 4-PBA. Conclusions: PCF inhibits UVB-induced apoptosis through restoring ER redox homeostasis, suppressing ER stress, and inhibiting ER stress-induced mitochondrial apoptosis in HaCaT cells. These findings provide evidence for the mechanism underlying UVB-induced skin damages, and support the promising role of PCF in treatment of the diseases.

**Keywords:** HaCaT cells, UVB irradiation, endoplasmic reticulum (ER), mitochondria, apoptosis

## Introduction

Ultraviolet radiation from the sun damages human skin, which would induce photoaging, inflammation, and even tumor formation [1, 2]. Particularly, ultraviolet B (UVB; from 290 nm to 320 nm) radiation could induce damage in epidermal keratinocytes of human skin, contributing to the development of skin cancers [3]. Recent studies have shown that UVB accumulates reactive oxygen species (ROS) production and initiates ROS-mediated mitochondrial apoptotic pathway in immortalized human keratinocyte cell lines [4, 5].

Polypeptide from *Chlamys farreri* (PCF) is a novel marine bioactive product isolated from

the Chinese scallop. Our previous studies demonstrate that PCF exerts protective effects in UVB-irradiated HaCaT cells, through scavenging ROS and inhibiting UVB-induced apoptosis [4, 5]. Mitochondria have been recognized as the main ROS producing sites in mammalian cells, which are also responsible for the initiation of apoptosis. However, it is well known that, in addition to mitochondria, endoplasmic reticulum (ER) has also been related to ROS generation and oxidative stress within cells [6]. It has been reported that UVB, besides its involvement in mitochondrial apoptosis initiation, could induce ER stress in HaCaT cells [7]. Excessively strong or long-term ER stress might lead to cell apoptosis, making itself another

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potential target of PCF [8, 9]. However, the effects of PCF on ER stress-induced apoptosis in keratinocytes under UVB irradiation have not yet been fully elucidated.

In this study, the ER-related protective effects of PCF in HaCaT cells subjected to UVB irradiation were investigated, and the relationship between ER stress and mitochondrial performance in UVB-irradiated HaCaT cells was also determined. Our results showed that PCF could inhibit UVB-induced apoptosis through restoring ER redox homeostasis, suppressing ER stress, and inhibiting ER stress-induced mitochondrial apoptosis in HaCaT cells.

### Materials and methods

#### *Materials and reagents*

Non-tumorigenic immortalized human keratinocyte HaCaT cells were kindly provided by Dr. Boxiao Ding (Yonsei University, Korea). PCF (96% purity) was kindly provided by Yellow Sea Fishery Research Institute (CAFS, Qingdao, Shandong, China). Cell culture DMEM medium, fetal bovine serum, penicillin, and streptomycin were purchased from Gibco (Gaithersburg, MD, USA). PDI, ERO-1, GRP78, and CHOP antibodies were purchased from Cell Signalling Technology (Beverly, CA, USA).  $\beta$ -actin antibody was purchased from Beijing Biosynthesis Biotechnology (Beijing, China). PI, JC-1, and BCA protein assay kits were purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). ECL Western blot kit was purchased from Pufei Biotechnology (Shanghai, China). Caspase fluorogenic substrates Ac-DEVD-AFC (for caspase-3) and Ac-LEHD-AFC (for caspase-9) were purchased from Enzyme Systems Products (Livermore, CA, USA).

#### *Cell culture and UVB irradiation*

HaCaT cells were cultured in DMEM medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. For PCF treatment, these cells were treated with 2.84 mM PCF for 2 h, and then subjected to the following treatments. For UVB irradiation, cell medium was replaced with D-Hanks' buffer, and cells were exposed to UVB at the dose of 20 mJ/cm<sup>2</sup> by UVB lamps with a peak emis-

sion at 302 nm (Beijing Normal University, Beijing, China). After UVB irradiation, cells were cultured with the original medium with PCF until analysis.

#### *MTT assay*

Cell viability was assessed by the MTT assay. Briefly, at the indicated time points, cells were incubated with 0.5 mg/ml MTT at 37°C for 4 h. The formazan crystals were extracted and dissolved in dimethyl sulfoxide (DMSO) at room temperature. Absorbance was read at 490 nm using a Molecular Devices VERSAmax microplate reader (Molecular devices, Sunnyvale, CA, USA).

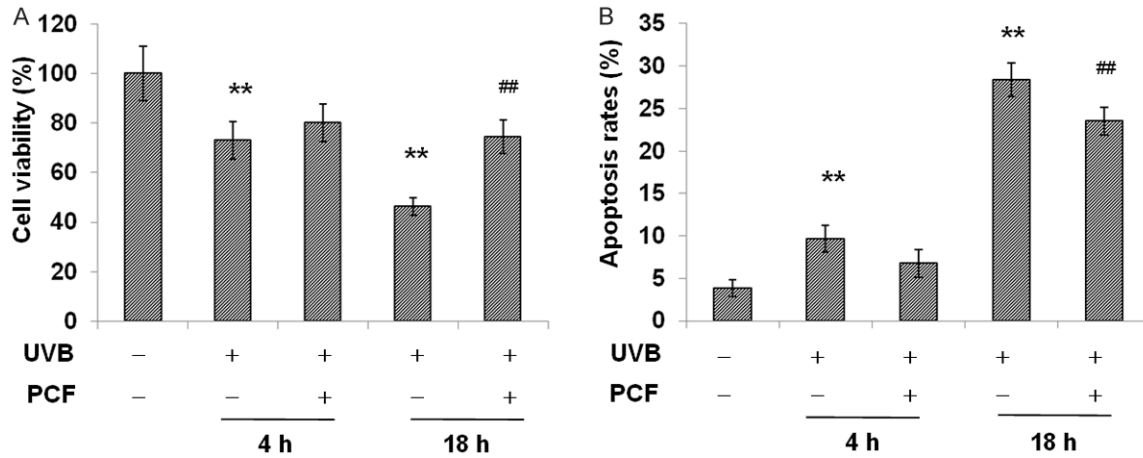
#### *Annexin V-FITC/PI staining and flow cytometry*

Apoptosis was analyzed using Annexin V-FITC/PI staining as previously described [10]. Briefly, cells were collected at the indicated time points and resuspended in 100 ml buffer containing 5 ml annexin V-FITC. Then 10 ml PI was added for incubation in dark at room temperature for 15 min. The apoptotic cells were analyzed by FACS-Vantage Flow Cytometer (Becton Dickinson and Company, San Jose, CA, USA).

#### *Western blot*

Western blot analysis was performed as previously described [11]. Briefly, cells were lysed on ice with lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mg/L leupeptin, and 1 mM PMSF). The lysate was centrifuged at 12000 g at 4°C for 10 min, and the protein concentration was determined with a BCA protein assay kit. 40  $\mu$ g protein was subjected to 12% SDS-polyacrylamide gel electrophoresis, and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% BSA in TBST at room temperature for 1 h, and then incubated with the primary antibodies against PDI, Ero-1 $\alpha$ , GRP78, CHOP, and  $\beta$ -actin (1:400 dilution) at 4°C overnight. The membrane was further incubated with second antibody (1:2000 dilution) at room temperature for 1 h. After washed with TBST for three times, protein bands were visualized using the ECL kit. The densities of sample bands were analyzed with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

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**Figure 1.** PCF increases cell viability and inhibits apoptosis in UVB-irradiated HaCaT cells. A. HaCaT cell viability at 4 h or 18 h after UVB irradiation, with or without PCF treatment. These cells were subjected to 20 mJ/cm<sup>2</sup> UVB irradiation. After 4 h or 18 h, the cell viability was assessed by MTT assay. B. Apoptosis rates of HaCaT cells at 4 h or 18 h after UVB irradiation, with or without PCF treatment. At 4 h or 18 h after UVB irradiation, the cell apoptosis rates were measured with annexin V-FITC/PI staining by flow cytometry. Compared with the control group, \*\**P* < 0.01; compared with the group without PCF treatment, ##*P* < 0.01.

### JC-1 staining

Mitochondrial membrane potential (MMP) was estimated with fluorescent probe JC-1. Briefly, the cells were trypsinized and stained with 2 mg/ml JC-1 in dark at 37°C for 20 min. After washed twice with PBS, the fluorescence at 488 nm was analyzed on a FACS-Vantage Flow Cytometer.

### Caspase activity assessment

Caspase fluorogenic substrates Ac-DEVD-AFC (for caspase-3) and Ac-LEHD-AFC (for caspase-9) were used to assess the caspase activities. Cells were harvested and resuspended in lysis buffer (1 mM EGTA, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 20 µg/ml leupeptin, 10 µg/ml pepstatin, 0.25% sodium deoxycholate, and 0.5% Nonidet P-40) on ice for 10 min. After three cycles of freeze-thaw, the lysate was centrifuged and the protein concentration was determined with the BCA protein assay kit. 50 µg protein was incubated with 100 µM caspase fluorogenic substrate, 25 mM HEPES-NaOH (pH 7.4), and 5 mM DTT at 37°C for 3 h. The cleaved amino-4-trifluoromethylcoumarin (AFC) products were measured on a Cytofluor 4000 fluorometer (PerSeptive Biosystems, Framingham, MA) using a 400 nm excitation filter and a 530 nm emission filter.

### Statistical analysis

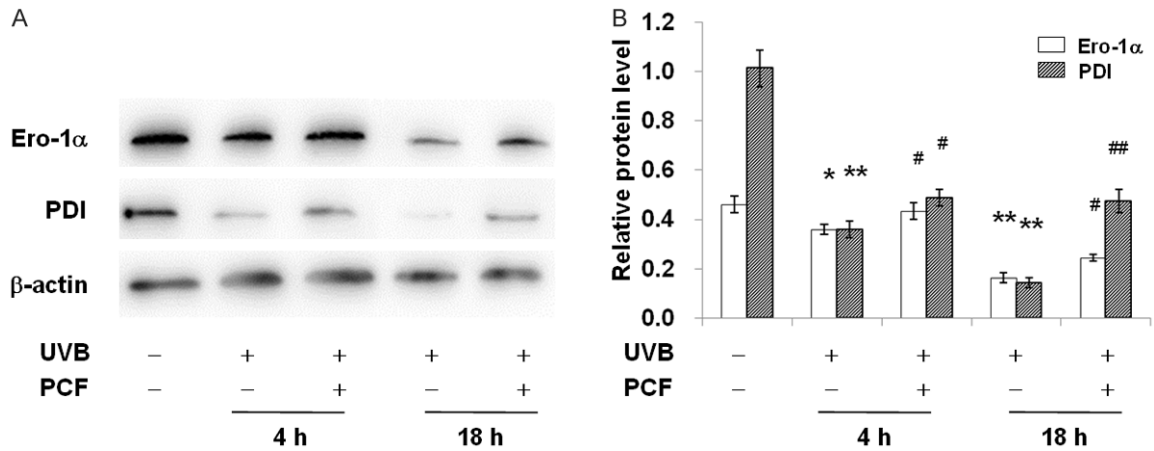
Data were expressed as mean ± SD. Statistical analysis was performed with SPSS 10.0 software. One-way analysis of variance and student's t-test were used for comparison. *P* < 0.05 was considered statistically significant.

### Results

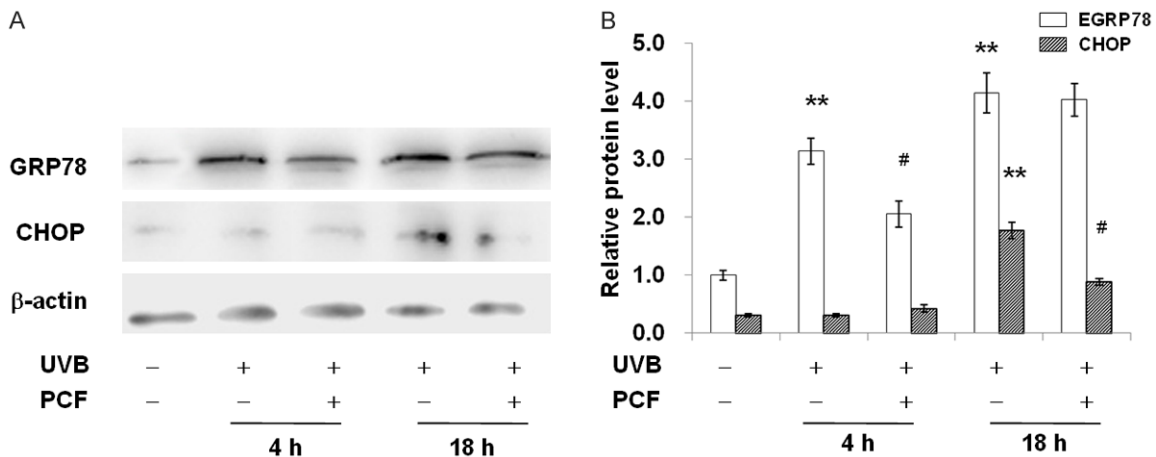
#### PCF increases cell viability in UVB-irradiated HaCaT cells

To investigate the effect of UVB irradiation on HaCaT cell proliferation, MTT assay was performed. Based on the pre-experiments, in HaCaT cells, apoptosis peaked at 18 h after 20 mJ/cm<sup>2</sup> UVB irradiation. In this study, HaCaT cells were subjected to 20 mJ/cm<sup>2</sup> UVB irradiation, and then subjected to the following measurements at 4 h (in the early stage) and 18 h (in the late stage), respectively, after irradiation. Results from the MTT assay showed that, compared to the control group, the cell viability declined to 73.5% at 4 h post-irradiation. After PCF treatment, the cell viability was slightly restored to 80.6% (**Figure 1A**). Furthermore, the cell viability was sharply decreased to 44.7% at 18 h after irradiation, which was obviously retrieved by PCF treatment (75.8%) (*P* < 0.01) (**Figure 1A**). These results suggest that PCF treatment could restore the declined

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**Figure 2.** PCF restores the ER redox homeostasis in UVB-irradiated HaCaT cells. A. The expression levels of PDI and Ero-1 in UVB-irradiated HaCaT cells were detected by Western blot analysis. B. Statistical analysis of the expression levels of PDI and Ero-1 in HaCaT cells. Compared with the control group, \* $P < 0.05$ , \*\* $P < 0.01$ ; compared with the group without PCF treatment, # $P < 0.05$ , ## $P < 0.01$ .



**Figure 3.** PCF suppresses ER stress in UVB-irradiated HaCaT cells. A. The expression levels of GRP78 and CHOP in UVB-irradiated HaCaT cells were detected by Western blot analysis. B. Statistical analysis of the expression levels of GRP78 and CHOP in HaCaT cells. Compared with the control group, \*\* $P < 0.01$ ; compared with the group without PCF treatment, # $P < 0.05$ .

cell viability in HaCaT cells following UVB irradiation.

### PCF protects against UVB-induced apoptosis in HaCaT cells

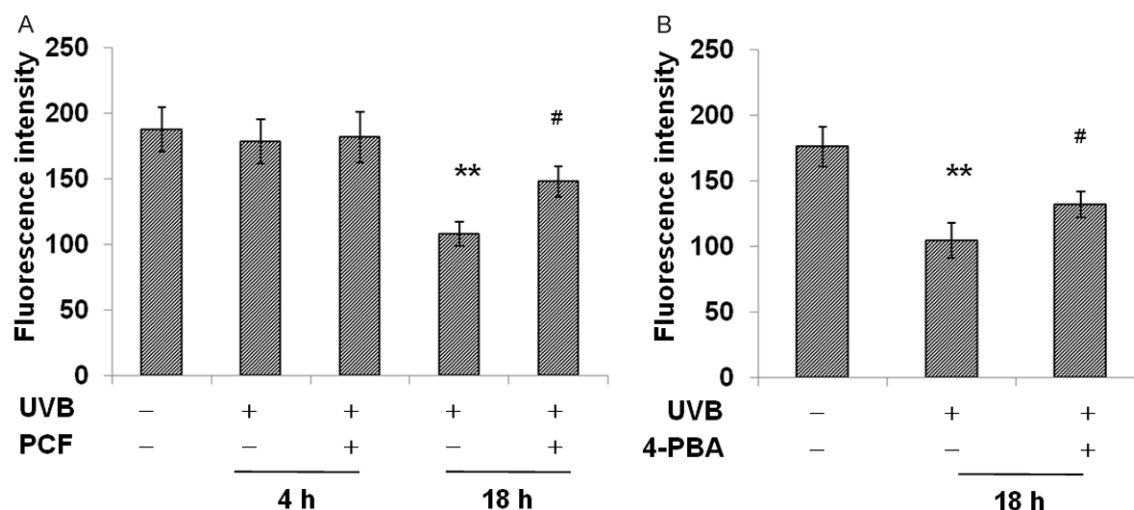
In addition to the cell viability, we further examined the effects of PCF treatment on apoptosis in HaCaT cells at 4 h and 18 h after UVB irradiation. Our results demonstrated that, compared with the apoptosis rate in the control group (4.38%), apoptosis was significantly increased in HaCaT cells at 4 h post-irradiation (9.09%) ( $P < 0.01$ ), which was further elevated to 28.22% at 18 h after irradiation ( $P < 0.01$ ) (Figure 1B).

PCF had no effects on apoptosis at 4 h after irradiation, while the treatment decreased the apoptosis rate to 22.15% at 18 h post-irradiation ( $P < 0.01$ ). These results indicate that UVB irradiation would enhance apoptosis in HaCaT cells, and PCF could reduce apoptosis in UVB-irradiated HaCaT cells.

### PCF restores ER redox homeostasis in UVB-irradiated HaCaT cells

Protein disulfide bond formation in ER is driven by protein disulfide isomerase (PDI) and endoplasmic reticulum oxidoreductin-1 (Ero-1), which are also closely linked with ER redox homeo-

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**Figure 4.** PCF alleviates UVB-induced MMP loss in HaCaT cells. A. MMP was measured with JC-1 staining in UVB-irradiated HaCaT cells, with or without PCF treatment. Measurement was performed at 4 h or 18 h after irradiation. B. The effects of 4-PBA on MMP were evaluated in UVB-irradiated HaCaT cells. Compared with the control group, \*\* $P < 0.01$ ; compared with the group without PCF treatment, # $P < 0.05$ .

stasis. To check the ER redox status in response to UVB irradiation in HaCaT cells, the expression levels of PDI and Ero-1 $\alpha$  was assessed with Western blot analysis. Our results showed that, compared with the control group, the expression levels of PDI and Ero-1 $\alpha$  were decreased at 4 h after irradiation, which dropped to an even lower level at 18 h post-irradiation. PCF treatment increased the expression of PDI and Ero-1 $\alpha$  in HaCaT cells after irradiation, at both 4 h and 18 h post-irradiation (Figure 2). These results indicate that, in HaCaT cells, ER redox imbalance occurred in the early stage after UVB irradiation, which is further deteriorated in the late stage following irradiation. PCF could restore the ER redox homeostasis in HaCaT cells after UVB irradiation.

### PCF suppresses ER stress in UVB-irradiated HaCaT cells

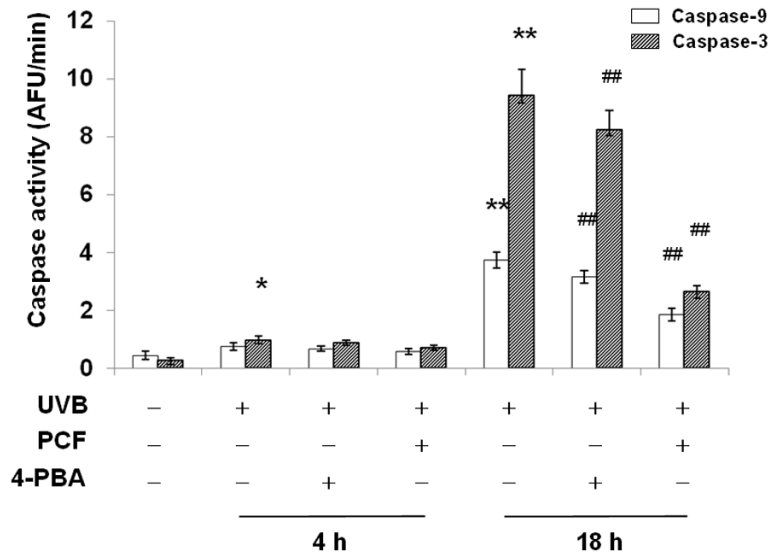
We next measured the expression levels of GRP78 and CHOP in UVB-irradiated HaCaT cells, which indicated ER stress and ER stress-induced apoptosis, respectively. As shown in Figure 3, our results showed that the expression level of GRP78 was increased dramatically in HaCaT cells at 4 h after UVB irradiation and further elevated at 18 h post-irradiation, which was in line with the time course of ER redox imbalance. On the other hand, the expression level of CHOP was not significantly changed in

HaCaT cells at 4 h after irradiation, which was, however, greatly increased at 18 h after irradiation, demonstrating that ER stress could induce apoptosis in the late stage after irradiation. PCF treatment inhibited the expression of GRP78 at 4 h after irradiation, and suppressed CHOP expression at 18 h post-irradiation. These results suggest that PCF could inhibit UVB-evoked ER stress in the early stage, and suppress the ER stress-induced apoptosis in the late stage after irradiation, in HaCaT cells.

### PCF alleviates UVB-induced mitochondrial membrane potential (MMP) loss in HaCaT cells

MMP plays a central role in the generation of reactive oxygen species (ROS) and mitochondrial apoptosis. We next examined the changes in MMP in HaCaT cells after UVB irradiation to investigate whether MMP dissipation was involved in ER stress. As shown in Figure 4, at 4 h after UVB irradiation, no obvious influence on MMP was observed in HaCaT cells. However, at 18 h after UVB irradiation, MMP was dramatically decreased, while PCF treatment alleviated the UVB-caused MMP loss, in HaCaT cells. These results indicate that MMP loss occurs later than ER stress, and PCF could contribute to the maintenance of MMP in the late stage of irradiation. When treated with the ER stress inhibitor, 4-PBA, the UVB-induced MMP loss was reversed in HaCaT cells. These results suggest that MMP loss might be the downstream

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**Figure 5.** PCF inhibits the activation of caspase-9 and caspase-3 in UVB-irradiated HaCaT cells. The activities of caspase-9 and caspase-3 were assessed in HaCaT cells at 4 h or 18 h after UVB irradiation, with or without PCF treatment. The effects of 4-PBA on the activities of caspase-9 and caspase-3 were also evaluated. Compared with the control group, \* $P < 0.05$ , \*\* $P < 0.01$ ; compared with the group without PCF treatment, ## $P < 0.01$ .

event of ER stress in HaCaT cells after UVB irradiation, and the inhibition of ER stress could, at least partly, alleviate the decline of MMP following irradiation in HaCaT cells.

### PCF inhibits caspase-9/-3 activation in UVB-irradiated HaCaT cells

Following MMP loss, caspase-9 could combine with Apaf-1 and ATP to process pro-caspase-3 to initiate mitochondrial apoptosis. Therefore, we continued to examine the activities of caspase-9 and caspase-3 in HaCaT cells after UVB irradiation. As shown in **Figure 5**, the level of caspase-9 was low at 4 h after UVB irradiation, with or without PCF and 4-PBA treatments. However, at 18 h after irradiation, the activity of caspase-9 was increased by 7.6 fold in HaCaT cells, which was significantly decreased by PCF and 4-PBA treatments ( $P < 0.01$ ). On the other hand, caspase-3 level started to increase at 4 h after irradiation, and achieved a 30.7-fold elevation at 18 h post-irradiation. The treatments of PCF and 4-PBA slightly decreased the activity of caspase-3 at 4 h after irradiation, and significantly declined the protein activity at 18 h post-irradiation (29.5% for PCF, and 85.3% for 4-PBA, of the peak level after UVB irradiation). These results suggest that caspase-9 is activated only in the late stage after UVB irra-

diation, while the activation of caspase-3 starts in the early stage post-irradiation and sustains through the late stage. Moreover, both PCF and 4-PBA could inhibit the activation of caspase-9 and caspase-3 in UVB-irradiated HaCaT cells.

### Discussion

In our previous studies, we have demonstrated that PCF exerts antioxidant and anti-apoptosis effects in UVB-irradiated HaCaT cells. The molecular mechanisms might involve mitochondria, CD95, NF- $\kappa$ b, COX-2, NOS/NO, and HSP90 [4, 5, 12-14]. The UVB-induced apoptosis model in HaCaT cells has been established, and the apoptosis rate peaks at 18 h post-irradiation

with the irradiation dose of 20-30 mJ/cm<sup>2</sup>. PCF treatment could inhibit apoptosis under UVB irradiation in HaCaT cells via maintaining MMP and depressing the activities of caspases, which suggest that PCF blocks UVB-induced mitochondrial apoptosis in HaCaT cells [4]. In the present study, the protective effects of PCF concerning ER in HaCaT cells after UVB irradiation were investigated, and the relationship between ER stress and mitochondrial performance in UVB-irradiated HaCaT cells was also detected.

Our results demonstrated that the cell viability started to decline at 4 h after UVB irradiation, and dropped greatly at 18 h post-irradiation. While the apoptosis rates in HaCaT cells were increased at 4 h after UVB irradiation, and dramatically elevated at 18 h post-irradiation. These results indicated that in the early stage after UVB irradiation, cell damages and apoptosis occurred, which continued to develop in the late stage after irradiation. In addition, MMP loss had been observed in the late stage after irradiation rather than in the early stage, suggesting that UVB-induced mitochondrial apoptosis might take place in the late stage. MMP loss could activate caspase-9 and caspase-3 to induce apoptosis. Our results showed that caspase-9 was activated only in the late stage

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after UVB irradiation, indicating that mitochondrial apoptosis occurred in the late stage rather than in the early stage after irradiation.

Caspase-3 activation was observed at 4 h post-irradiation and further enhanced at 18 h post-irradiation. As caspase-9 is not the exclusive activator of caspase-3, these results indicate that other pathways might be involved in the early stage of UVB-induced apoptosis, for which more in-depth studies are still needed. Although PCF inhibited UVB-induced apoptosis in the late stage through maintaining MMP and suppressing caspase-9 and caspase-3, in the early stage PCF only exerted slight protective effects on cells.

ER is a cellular organelle involved in the synthesis and processing of proteins. PDI and Ero-1 are the major enzymes that maintain the ER redox homeostasis. The disruption of the redox balance might lead to protein misfolding, which would result in ER stress. In response to ER stress, cells activate unfolded protein response (UPR). During UPR initiation, GRP78 preferentially binds to the unfolded proteins, driving its equilibrium binding away from IRE-1, PERK, and ATF-6 proteins which are initiators of the three main signalling cascades of UPR [9]. Our results from PDI and Ero-1 expression detection indicated that ER redox homeostasis was disturbed shortly after UVB irradiation. In addition, the increased expression of GRP78 was observed at both 4 h and 18 h post-irradiation. Previous studies indicate that GRP78 is dissociated from the ER stress transducers to activate UPR transcription, and the dissociation of GRP78 might exist throughout the whole period after irradiation. If ER stress is prolonged or overwhelming, UPR will fail to maintain normal ER function, and the adaptive UPR will switch to pro-apoptotic signals, such as CHOP, to eliminate the irreversibly damaged cells [8, 15]. Our results showed that the expression of CHOP was increased only at 18 h post-irradiation, suggesting that ER stress occurred in the late stage after irradiation, which was overwhelming and resulted in apoptosis. PCF treatment could inhibit GRP78 in the early stage after irradiation, and depress CHOP expression in the late stage, suppressing UVB-induced ER stress and the related apoptosis.

Due to the close relationship between mitochondria and ER, we recognized ER as a possi-

ble target for PCF. Our results showed that both mitochondria and ER participated in the UVB-induced apoptosis in HaCaT cells. It has been well accepted that a burst of oxidative stress in ER will target mitochondria, resulting in excessive production of ROS and subsequent apoptosis [6]. In this study, with the ER stress inhibitor 4-PBA, we identified the association between mitochondria and ER in UVB-irradiated HaCaT cells. MMP loss occurred in the late stage post-irradiation, and 4-PBA could restore MMP loss, suggesting that ER acted upstream of mitochondria in UVB-irradiated HaCaT cells. Moreover, after 4-PBA treatment, the activities of caspase-9 and caspase-3 were declined in the late stage after irradiation rather than in the early stage, which was in line with the changes in MMP, indicating that ER stress initiated mitochondrial apoptosis in UVB-irradiated HaCaT cells. However, ER stress seemed not to be the only inducer of mitochondrial apoptosis. PCF depressed caspase-9 and caspase-3 to a lesser extent than 4-PBA, probably because PCF inhibited UVB irradiation-induced apoptosis not only through suppressing ER stress but also through interacting with other possible targets.

In conclusion, our results showed that PCF alleviated cell viability decline and inhibited apoptosis in HaCaT cells after UVB irradiation. Moreover, PCF restore the ER redox homeostasis, and inhibited UVB-evoked ER stress and apoptosis, in HaCaT cells following UVB irradiation. In addition, PCF alleviated the UVB-induced MMP loss, and inhibited the activation of caspase-9 and caspase-3. On the other hand, MMP loss and caspase-9/-3 activation could be partly blocked by ER stress inhibitor 4-PBA. PCF inhibits UVB-induced apoptosis through restoring ER redox homeostasis, suppressing ER stress, and inhibiting ER stress-induced mitochondrial apoptosis in HaCaT cells. These findings provide evidence for the mechanism underlying UVB-induced skin damages, and support the promising role of PCF in the treatment of the diseases.

### Acknowledgements

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**Disclosure of conflict of interest**

None.

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