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Ultra-violet B (UVB)-induced skin cell death occurs through a cyclophilin D intrinsic signaling pathway

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ABSTRACT

UVB-induced skin cell damage involves the opening of mitochondrial permeability transition pore (mPTP), which leads to both apoptotic and necrotic cell death. Cyclophilin D (Cyp-D) translocation to the inner membrane of mitochondrion acts as a key component to open the mPTP. Our Western-Blot results in primary cultured human skin keratinocytes and in HaCaT cell line demonstrated that UVB radiation and hydrogen peroxide (H_2O_2) induced Cyp-D expression, which was inhibited by anti-oxidant *N*-acetyl cysteine (NAC). We created a stable Cyp-D deficiency skin keratinocytes by expressing Cyp-D-shRNA through lentiviral infection. Cyp-D-deficient cells were significantly less susceptible than their counterparts to UVB- or H_2O_2-induced cell death. Further, cyclosporine A (Cs-A), a Cyp-D inhibitor, inhibited UVB- or H_2O_2-induced keratinocytes cell death. Reversely, over-expression of Cyp-D in primary keratinocytes caused spontaneous keratinocytes cell death. These results suggest Cyp-D's critical role in UVB/oxidative stress-induced skin cell death.

1. Introduction

The ultraviolet radiation (UVR) is the major environmental factor that affects the functions and survival of many skin cell types. Excessive UVR contributes to skin cancers such as basal cell carcinoma, squamous cell carcinoma and malignant melanoma [1–3]. Acute responses of human skin to UVR include photo-damage, erythema, mutation, immune-suppression, vitamin D synthesis and tanning. Chronic UVR effects include immune-suppression, photo-aging and photo-carcinogenesis [1–3]. The UVR that reaches human skin consists mainly of long wavelength UVA (320–400 nm), together with only a minority of short wavelength UVB (280–320 nm) [3]. UVC (200–280 nm) is screened out by atmospheric oxygen through the ozone layer absorption [4]. Although the amount of UVB is much less than UVA (estimated at 5%) in UVR that reaches human skin, it is more cytotoxic and mutagenic than UVA, and is 3–4 orders of magnitude more effective per unit physical dose (J cm^{-2}) than UVA for DNA damage [1–4]. As such, our group has been focusing on the molecular mechanisms of UVB-induced skin damage [5–8].

The mitochondrial permeability transition pore (mPTP) plays a pivotal role in both necrotic and apoptotic neuronal cell death. Various stress conditions open mPTP to cause the membrane potential collapses, which induces apoptotic cell death by releasing proteins from the inner membrane space [9–11]. Cyclophilin D (Cyp-D), a peptidylprolyl isomerase, resides in the mitochondrial matrix and associates with the inner mitochondrial membrane [12–14]. Studies have confirmed that oxidative and other cellular stresses promote Cyp-D translocation to the inner membrane of mitochondrion, which triggers the opening of the mPTP and cell death [12–14]. And a genetic deficiency of CypD inhibits mPTP opening and protects from Ca^{2+}- and oxidative stress-induced cell death [15–17]. In the current study, we are set to understand the potential role of Cyp-D in UVB-induced skin cell damage.

2. Materials and methods

2.1. Chemicals and reagents

Cyclosporine A (CsA) and tumor-necrosis factor-α (TNF-α) were obtained from Sigma (Sigma, St. Louis, MO); Anti-Erk1/2, tubulin,

Abbreviations: Cyp-D, cyclophilin D; Cs-A, cyclosporine A; H_2O_2, hydrogen peroxide; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; NAC, *N*-acetyl cysteine; TNF-α, tumor-necrosis factor-α; UVB, ultraviolet B.

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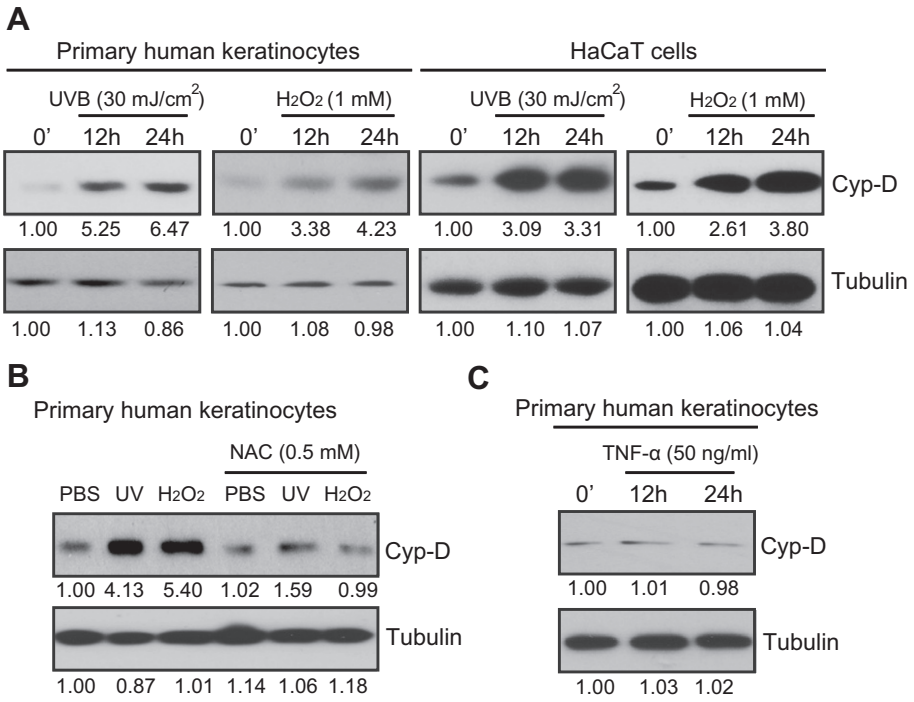

Fig. 1. UVB radiated skin keratinocytes show Cyclophilin D upregulation. Representative Western-Blots showing the expression of Cyclophilin D (Cyp-D) and tubulin (equal loading) in primary cultured human skin keratinocytes and HaCaT cell line after indicated UVB radiation or H_2O_2 treatment (A). Representative western-blots showing the expression of Cyp-D and tubulin in UVB (30 mJ/cm^2, 24 h) or H_2O_2 (0.5 mM, 24 h) treated skin keratinocytes, with or without NAC pretreatment (0.5 mM, 1 h pretreatment) (B). Representative Western-Blots showed the expression of Cyp-D and tubulin in primary cultured skin keratinocytes with indicated TNF-α treatment (C). The blots in this figure were quantified by Image J software. Experiments in this figure were repeated three times to insure consistency of results.

rabbit/mouse IgG-horseradish peroxidase (IgG-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cyp-D shRNA (sc-44892-V) and scramble shRNA (sc-108080) lentiviral particles were obtained from Santa Cruz Biotech (Santa Cruz, CA).

2.2. Cell culture and UVB radiation

Primary human skin keratinocytes (ATCC, PCS-200-010, Beijing, China) were maintained in a Dermal Cell Basal Medium (ATCC, PCS-200-030, Beijing, China), supplemented with a Keratinocyte Growth Kit (ATCC PCS-200-040, Beijing, China), penicillin/streptomycin (1:100, Sigma, St. Louis, MO), in a CO_2 incubator at 37 °C. HaCaT keratinocytes cell line was cultured as previous reported [6,8]. UVB radiation equipments and procedure were described in [8,18].

2.3. Live cell counting by trypan blue staining

As previous reported [8,18], the total number of viable skin keratinocytes (trypan blue positive) after indicated treatment/s was counted, and the percentage (%) of viable cells was calculated by the number of the trypan blue stained cells divided by the total number of the cells.

2.4. Clonogenicity assay

Primary cultured skin keratinocytes (1×10^4) were suspended in 1 ml of culture medium (ATCC, PCS-200-030+040, Shanghai, China) and with indicated treatments or vehicle controls. The cell suspension was then added on top of a pre-solidified 1% agar in a 100 mm culture dish. The medium was replaced every two days. After 8 days of incubation, colonies were photographed at 4×. Colonies larger than 50 μm in diameter were counted.

2.5. Generation of Cyp-D knockdown stable skin keratinocytes by lentiviral shRNA transfection

Cyp-D shRNA containing lentiviral particles (20 μl/ml) were added to primary cultured human skin keratinocytes for 48 h. Afterwards, puromycin (2 μg/ml)-containing fresh medium was added every 2–3 days until resistant stable cells were formed. The expression level of CHOP was detected by Western-Blots. Only Cyp-D deficient (knockdown) stable cells were selected for further experiments. Same amount of scramble shRNA lentiviral particles were added in control cells.

2.6. Cyp-D vector construction and transfection

Cyclophilin D cDNA was PCR amplified from a PC12 cell cDNA library using a pair of specific primers (5′-GCA CCGAATTCATGC-TAGCTCTGC-3′ and 5′-GGCTTGAATTCTTAGCTCAACTGGCC-3′) to introduce EcoRI flanking linkers before and after the CypD coding sequence. The fragment was cut with EcoRI (Invitrogen) and ligated into the EcoRI site of the pSuper-puromycin vector (Clontech). The insertion and correct orientation of CypD was verified by PCR and restriction mapping. Lipofactamine (Invitrogen) protocol was used to transfect vector or the plasmid [6,19,20]. Stable cells were selected by puromycin. The resulting pSuper-CypD construct was subjected to Western-Blots detecting Cyp-D expression in stable cells.

Western Blot, analysis of cell death by propidium iodide (PI) fluorescence-activated cell sorting (FACS), and cell viability assay ("MTT" assay) were described in our previous studies [6,19–21].

2.7. Statistical analysis

Individual culture dishes or wells were analyzed separately. In each experiment a minimum of three wells/dishes of each treat-

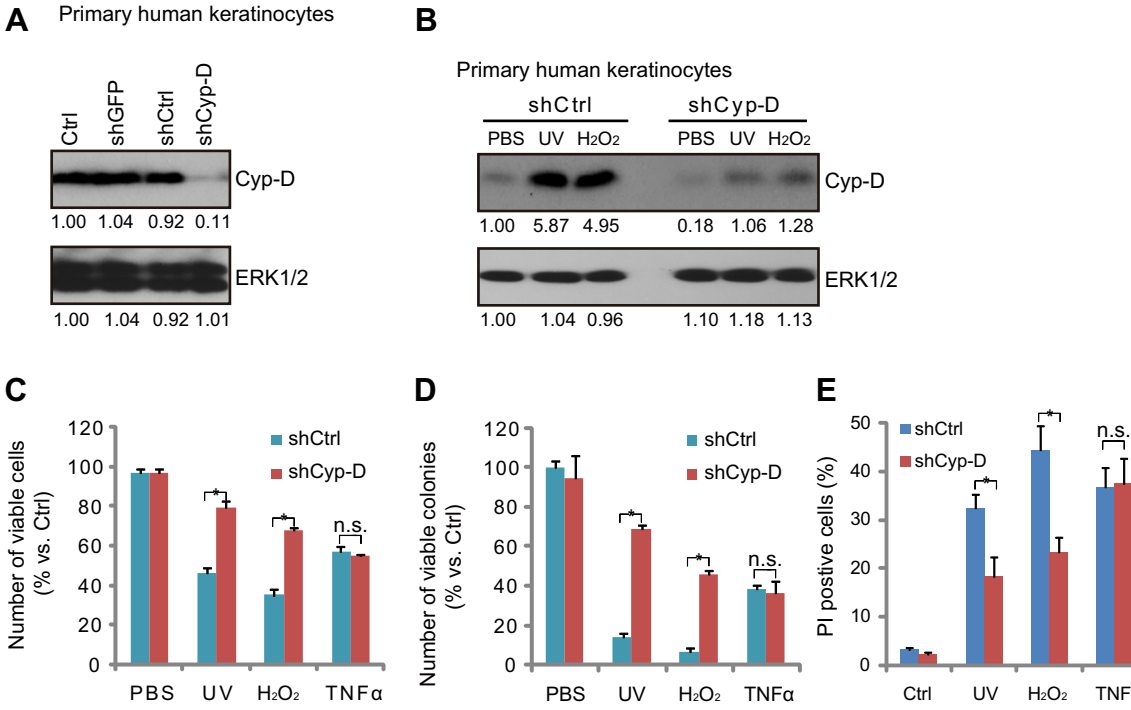

Fig. 2. UVB-induced skin keratinocytes death is diminished after Cyp-D silencing. Representative western-blots showing the expression of Cyp-D and ERK1/2 (equal loading) in primary cultured human skin keratinocytes transfected with lentiviral particle-packed scramble, green fluorescence protein (GFP), or Cyp-D shRNA (A). Representative Western-Blots showing the expression of Cyp-D and tubulin after UVB (UV, 30 mJ/cm^2, 24 h) or H_2O_2 (0.5 mM, 24 h) treatments in stable primary cultured human skin keratinocytes expressing control (scramble, sc) or Cyp-D shRNA (B). Stable primary cultured keratinocytes expression control shRNA (scramble) or Cyp-D shRNA were treated with UVB (UV, 30 mJ/cm^2), H_2O_2 (0.5 mM) or TNF-α (50 ng/ml), cell death was analyzed by trypan blue staining (C, after 48 h), clonogenicity assay (D, after 8 days) and FACS sorting PI positive cells (E, after 48 h). The blots in this figure were quantified by Image J software. Experiments in this figure were repeated three times to insure consistency of results. $^*P < 0.05$ (ANOVA). N.s.: no statistical difference.

ment was used. Each experiment was repeated a minimum of three times. All data were normalized to control values of each assay and are presented as mean ± standard deviation (SD). Data were analyzed by one-way ANOVA followed by a Scheffe's *f*-test by using SPSS software (SPSS Inc., Chicago, IL, USA). Significance was chosen as $P < 0.05$.

3. Results

3.1. UVB radiated skin keratinocytes show cyclophilin D (Cyp-D) upregulation

The main focus of this study is to test the potential role of Cyp-D in UVB-induced skin cell damage. Western-Blot results in Fig. 1A demonstrated that UVB radiation induced a significant Cyp-D upregulation in primary cultured skin keratinocytes and HaCaT cell line. Anti-oxidant *N*-Acetyl-Cysteine (NAC) significantly inhibited UVB-induced Cyp-D expression (Fig. 1B). Meanwhile, H_2O_2 mimicked UVB's effect and promoted Cyp-D expression (Fig. 1A). These results suggested that reactive oxygen species (ROS) might be the key factor to mediate Cyp-D expression by UVB. Results in Fig. 1C demonstrated TNF-α had almost no effect on Cyp-D expression in primary cultured skin keratinocytes.

3.2. UVB-induced skin keratinocytes death is diminished after Cyp-D silencing

As described in material and methods, we successfully created a stable primary skin keratinocytes line expressing Cyp-D shRNA. Western-Blot results in Fig. 2A showed that Cyp-D expression was deficient in the stable cells (11% of control cells). Further, UVB and H_2O_2-induced Cyp-D expression was also largely inhibited (Fig. 2B). Trypan blue staining, clonogenicity assay and PI FACS were used to test skin cell death as described. We found that UVB and H_2O_2-induced skin keratinocytes cell death was inhibited in Cyp-D deficient cells (Fig. 2C–E). Note that the TNF-α-induced keratinocytes cell death was not affected (Fig. 2C–E). The number of viable skin keratinocytes after UVB (UV, 30 mJ/cm^2, 48 h) radiation increased from 46.0 ± 2.6% to 79.1 ± 3.9% in Cyp-D deficient cells (Fig. 2C) ($P < 0.05$), while the percentage of PI positive cells decreased from 32.1 ± 3.2% to 18.4 ± 3.7% (Fig. 2E) ($P < 0.05$).

3.3. Cyclosporine A (CsA) inhibited UVB/H_2O_2-induced skin keratinocytes death

Results in Fig. 3 demonstrated that Cyp-D inhibitor CsA (10 μM) significantly inhibited UVB/H_2O_2-induced primary skin keratinocytes cell death (Fig. 3B and C). Note that CsA didn't affect UVB and H_2O_2-induced Cyp-D expression (Fig. 3A). The number of viable skin keratinocytes after UVB (UV, 30 mJ/cm^2, 48 h) radiation increased from 48.2 ± 3.4% to 72.3 ± 5.3% with CsA co-treatment (Fig. 3B) ($P < 0.05$), while the percentage of PI positive cells decreased from 28.2 ± 3.7% to 16.3 ± 2.3% (Fig. 3C) ($P < 0.05$). These results confirmed the gene silencing data in Fig. 2 and further suggested the requirement of Cpy-D in UVB-induced skin cell death.

3.4. Overexpression of Cyp-D caused spontaneous cell death in primary cultured keratinocytes

To further investigate the role of Cyp-D in keratinocytes cell death, we created a stable primary cultured skin keratinocytes line with Cyp-D overexpression (see Section 2). Western-Blots results of Fig. 4A confirmed Cyp-D over-expression in the cell line. Cells with Cyp-D over-expression showed spontaneous cell death as evi-

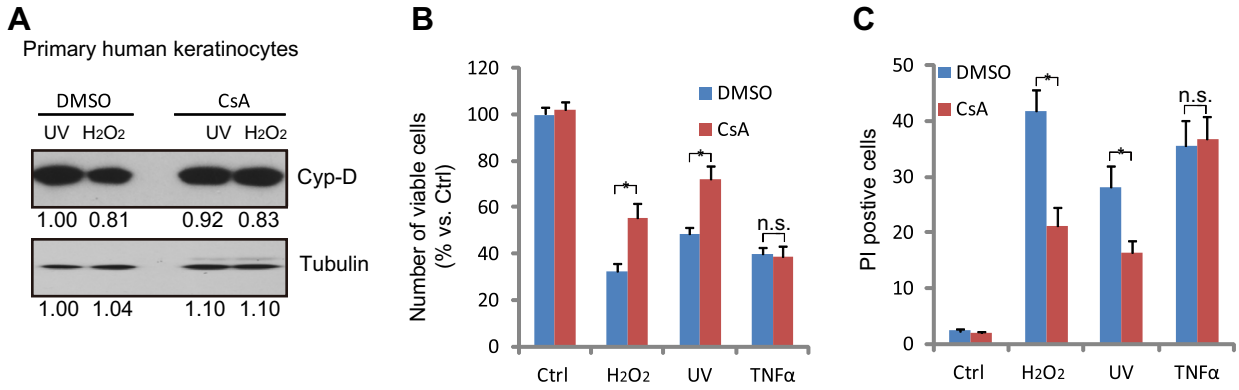

Fig. 3. Cyclosporine A (CsA) inhibited UVB/H_2O_2-induced skin keratinocytes death. Primary cultured human skin keratinocytes were pre-treated with CsA (10 μM, 30 min pretreatment), followed by UVB (UV, 30 mJ/cm^2) or H_2O_2 (0.5 mM) treatment, expression of Cyp-D and tubulin was analyzed by Western-blot 24 h later, and cell death was analyzed by trypan blue staining (B), and FACS sorting PI positive cells (C) after 48 h. The blots in this figure were quantified by Image J software. Experiments in this figure were repeated three times to insure consistency of results. *$P < 0.05$ (ANOVA).

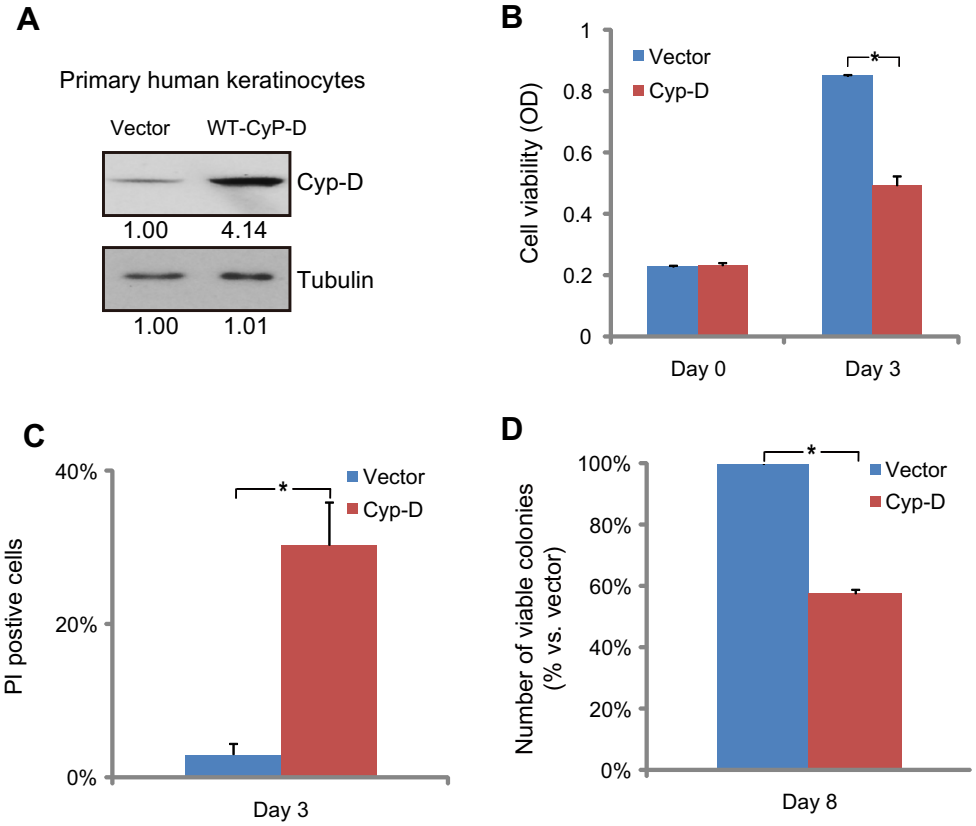

Fig. 4. Overexpression of Cyp-D caused spontaneous primary keratinocytes cell death. Representative western-blots showing the expression of Cyp-D and tubulin in stable primary cultured human skin keratinocytes transfected with Vector (pSuper-puro) or Cyp-D-cDNA (pSuper-puro-Cyp-D) (A). Same number of Vector (pSuper-puro)- and Cyp-D-(pSuper-puro-Cyp-D) transfected stable human skin keratinocytes were subjected to MTT cell viability assay (B), FACS sorting PI positive cells (C) and the clonogenicity assay (D) to test cell death. The blots in A were quantified by Image J software. Experiments in this figure were repeated three times to insure consistency of results. *$P < 0.05$ (ANOVA).

denced by less cell viability (Fig. 4B), more PI positive cells (Fig. 4C), and less colonies formation (Fig. 4D), compared to the vector transfected control cells.

4. Discussion

Mitochondrion is critical for both apoptotic and necrotic cell death. The opening of mPTP leads to mitochondrial swelling, outer membrane rupture and the release of apoptotic mediators (cytochrome C for example) [22]. It is known that the mPTP is composed of the adenine nucleotide translocator (ANT) on the mitochondrial inner membrane, a voltage-dependent anion channel (VDAC) on the mitochondrial outer membrane, and Cyp-D in the mitochondrial matrix [22]. Knockout studies in transgenic mice have shown that Cyp-D is critical for the function of mPTP and cell death [23,24]. Cyp-D mice were resistant against ischaemia/reperfusion-induced cell death *in vivo*, whereas mice overexpressing Cyp-D demonstrate spontaneous cell death with mitochondrial

swelling [23,24]. Further, *in vitro* evidences suggest that cells isolated from Cyp-D mice are significantly protected from Ca^{2+}-overload or oxidative stress-induced cell death, but not TNF-α-regulated cell death [23,24].

In the current study, we found that both UVB radiation and H_2O_2 treatment significantly induced Cyp-D expression in both primary cultured human skin keratinocytes and HaCaT cell line. Inhibition of Cyp-D by gene silencing or by CsA significantly reduced UVB-induced cell death. Meanwhile, overexpression of CypD in primary cultured skin keratinocytes caused spontaneous cell death. These results suggest that Cyp-D is important for UVB-induced keratinocytes cell death, and inhibition of CypD should be protective against UVB-induced skin cell damage.

Based on this current study, we suggest that Cyp-D could serve as a potential drug target for UV-induced skin cell damage. Also our study here show that Cyp-D inhibitor cyclosporine A protects UV-induced skin cell damage, it has been found to be clinically toxic because of its immunosuppressive effect. Hence, there is a need for non-toxic cyclosporine A analogs that may prove effective and selective for skin aging treatment.

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