Nrf2- and Bach1 May Play a Role in the Modulation of Ultraviolet A-Induced Oxidative Stress by Acetyl-11-Keto- β -Boswellic Acid in Skin Keratinocytes

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Keywords

Ultraviolet A irradiation · Skin keratinocytes · Acetyl-11-keto- β -boswellic acid · Nrf2 · Heme oxygenase-1 · Bach1

Abstract

Background: Exposure of human skin to solar ultraviolet A (UVA) irradiation causes severe oxidative stress with damage to various cellular components and concomitant inflammation and carcinogenesis. **Objective:** The aim of this study is to investigate the protective effect of acetyl-11-keto- β -boswellic acid (AKBA) against UVA radiation on human skin keratinocytes. **Methods:** HaCaT cells were pretreated with AKBA followed by UVA irradiation. Radiation effects on cell morphology, cell viability, intracellular reactive oxygen species (ROS) levels, and antioxidant enzymes were examined. **Results:** AKBA reduces UVA irradiation-induced cell viability loss, accompanied by a decreased production of UVA-induced ROS, decreased malondialdehyde, and increased superoxide dismutase expression. In addition, AKBA increased basal and UVA-induced levels of Nrf2 (NF-E2-related factor 2), the redox-sensitive factor, and its target genes NQO1 and heme oxygenase-1 (HO-1), whereas expression of the tran-

scriptional repressor Bach1 (BTB and CNC homology 1) was reduced. Furthermore, the cytoprotective effects of AKBA against UVA-derived oxidative damage were accompanied by modulating expression of inflammatory mediators (i.e., cyclooxygenase-2 and nuclear factor- κ B) and NOX1. **Conclusions:** AKBA protects skin cells from UVA-induced damage by modulating inflammatory mediators and/or ROS production. Therefore, AKBA has potential in the development of skin care products.

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Introduction

It is known that solar ultraviolet (UV) irradiation of human skin leads to photoaging, immunosuppression, inflammation, and ultimately carcinogenesis [1–3]. Ultraviolet A (UVA) comprises the largest portion (>95%)

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of the solar UV radiations reaching the biosphere, and is also a predominant source to trigger oxidative stress in human skin [1, 4]. UVA exposure of cells generates reactive oxygen species (ROS) by complex photochemical interactions with intracellular chromophores so that concomitant oxidative stress occurs in the irradiated tissues [5]. Prolonged UVA exposure may destroy the skin's natural defense system and cause oxidative damage or an inflammatory response that can be mediated by cyclooxygenase-2 (COX-2) and nuclear factor- κ B (NF- κ B) [6, 7]. Previous studies reported that acetyl-11-keto- β -boswellic acid (AKBA) hinders the induction of NF- κ B and COX-2-mediated signaling pathways, and reduced intestinal carcinogenesis [7–10]. Furthermore, AKBA behaves like an antioxidant that modulates the expression of stress-responsive proteins like HO-1 via the Nrf2 pathway, and therefore has a neuronal protection function [7].

Physiological redox homeostasis is achieved by a variety of cytoprotective strategies which have evolved for cellular adaptation against UVA-induced oxidative damage [9]. Once cellular redox homeostasis is markedly disturbed by continuous oxidative stress, the imbalance between malondialdehyde (MDA), superoxide dismutase (SOD), and iron molecules will lead to oxidative damage and promote photoaging and skin cancer [11–14]. Importantly, UVA also increases the levels of certain oxidizing enzymes such as NADPH oxidase 1 (NOX1), a major producer of superoxide anion (O_2^-) in UVA-irradiated skin keratinocytes. Therefore, inhibition of NOX1 by diphenyleneiodonium chloride (DPI) protects irradiated cells [9, 15].

To defend against oxidative stress, the antioxidant CNC (cap “n” collar) family transcription factor Nrf2 (NF-E2-related factor 2) translocates into the cell nucleus to form a functional heterodimer with a small Maf protein to bind to antioxidant response element (ARE) sequences in target gene promoter regions [16, 17], which in turn leads to expression of ARE-driven cytoprotective genes, including those encoding antioxidant and detoxification enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione S-transferases P1 (GSTP1) [16]. Under basal conditions (sham or nonirradiation), Nrf2 is sequestered in the cytoplasm by being tethered to an actin-binding Kelch-like ECH-associating protein 1 (Keap1), and Nrf2 levels increased in Keap1^{-/-}-deficient mice [17, 18]. However, transactivation of Nrf2 can be inhibited by another CNC transcription repressor, Bach1 (BTB and CNC homology 1), through binding to ARE sequences in target

genes [19, 20]. Nrf2 has been found to protect human keratinocytes and mouse dermal fibroblasts from UVA irradiation-mediated damage [21, 22].

Activation of the Nrf2-ARE-driven gene regulatory pathway by a variety of natural compounds confers chemoprevention against various diseases of oxidative stress origin [17, 23]. This pathway is considered a promising therapeutic target for natural phytochemicals derived from various natural products. Chemoprevention by phenolic compounds and flavonoids, such as quercetin, rutin, black tea polyphenols, curcumin, and sulforaphane, occurs mainly by evoking Nrf2-directed antioxidant and anti-inflammatory responses [12]. AKBA is one of the active constituents of gum resin of the medicinal plant *Boswellia serrata* (Roxb. ex Colebr) and has long been used for treatment of inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease [24, 25].

Since AKBA can reduce NF- κ B and COX-2 levels, it can therefore inhibit inflammations [7–10] caused either by oxidative stress or any other stressors. Thus, we questioned if AKBA has inhibitory effects on UVA-induced inflammation and oxidative damage or if it elicits through Nrf2- and Bach1-directed ARE-dependent gene regulatory networks in human skin keratinocytes. Therefore, in the present study, we investigated the photoprotective properties of AKBA in human skin keratinocytes HaCaT and revealed that AKBA exerts a dual opposing effect on Nrf2- and Bach1-driven antioxidant pathways in the cytoprotective response against UVA-derived oxidative stress and downregulates inflammatory factors.

Materials and Methods

Antibodies, Chemicals, and Other Reagents

Fetal bovine serum (FBS, TBD21HY), RPMI 1640 medium (HyClone), penicillin, and streptomycin were purchased from North China Pharmaceutical Co., Ltd. (China). Antibodies against Nrf2 (H-300, sc-13032), Bach1 (F-9, sc-271211), and NQO1 (C-19, sc-16464) were obtained from Santa Cruz Biotechnology Inc. (USA). The polyclonal antibodies against GSTP1 (3F2) and HO-1 (ADI-OSA-110-F) were purchased from Cell Signaling Technology and ENZO Life Sciences, respectively. The anti- β -actin antibody along with the secondary antibodies against goat and rabbit IgG were bought from Beijing Zhong Shan-Golden Bridge Biological Technology Co. Ltd., while the secondary antibodies against mouse IgG were obtained from Beyotime (China). AKBA, with a purity of more than 99%, was obtained from ENZO Life Sciences. 3-[4, 5-dimethyl-2-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma (USA). Additional commercial kits used to measure MDA and SOD were obtained from the Jiancheng Institute of Biotechnology (Nanjing, China).

Pretreatment of Cells with AKBA Following UVA Irradiation

Immortalized human skin keratinocytes (HaCaT) and mouse embryonic fibroblasts, including wild-type cells (WT) and Keap1 knockout cells (Keap1^{-/-}), were cultured in RPMI 1640 medium as previously reported [21, 22]. Cells were pretreated with 0.125–2.5 μM of AKBA for 12 h and were then exposed to UVA irradiation at the indicated doses from 80 to 400 kJ/m^2 [24, 25] by using a broad-spectrum UV lamp (UVB was blocked with an appropriate filter provided with the instrument) at the peak wavelength of 365 nm (MUA-165, Beijing Normal University, Beijing, China). A trial dose of UVA irradiation at 160 kJ/m^2 caused 22% cell death, i.e., the surviving 78% cells that were used for the following experimental analyses.

Cell Viability Measured by the MTT Assay

Cells (8×10^3) were cultured in each well of 96-cell plates for 24 h and then pretreated with AKBA at the indicated concentrations for an additional 2–24 h. Then cells were irradiated by the indicated doses of UVA. The cells were allowed to recover from irradiation for 12 h in a medium supplemented with 1% (v/v) heat-inactivated FBS, and were then incubated with a medium containing MTT for additional 2 h. A microplate reader (Model 680, Bio-Rad) was used for measurement of cell viability at 490 nm. The effect of AKBA on cell viability was calculated as the percent of viable cells compared with the vehicle control cells.

Western Blot Analysis

The treated cells were washed with ice-cold phosphate-buffered saline (PBS), and the whole-cell extracts were obtained and blotted with primary antibodies against Nrf2 (1:200), NQO1 (1:200), HO-1 (1:200), GSTP1 (1:1,000), NOX1 (1:500), Bach1 (1:200), COX-2 (1:500), NF- κB (1:1,000), or β -actin (1:1,000; as an internal loading control), and with a secondary anti-IgG antibody (1:1,000). The immunoblots were visualized by the Super Signal West Pico chemiluminescence (ECL) substrate (Rockford, IL, USA) and imaged by the Versa DocTM Imaging System.

Immunofluorescence of Nrf2

HaCaT cells (2.5×10^4) in a 48-well plate were pretreated with AKBA at 1.0 μM for 12 h prior to 160 kJ/m^2 UVA irradiation. Then the cells were incubated overnight with the primary anti-Nrf2 (1:100) antibody at 4°C. The specimens were washed with PBS and incubated with AlexaFluor 488-conjugated secondary antibody (1:500) for 1 h, and stained by 1 $\mu\text{g}/\text{mL}$ of DAPI (Roche) for 10 min. Subsequently, the stained cells were visualized with a fluorescence microscope (BX51; Olympus, Japan). For cell shape observation, the cells were incubated with fluorescein isothiocyanate (FITC) for 1 h at room temperature (RT) and then stained with 1 $\mu\text{g}/\text{mL}$ of DAPI for 10 min.

Gene Expression Analysis

To quantify gene expression levels, real-time qPCR experiments were carried out using the Promega GoTaq[®] qPCR Master Mix (A6001) in a LightCycler apparatus (C1000 Touch, Bio-Rad). Specific primers were designed as follow: *hHO-1* (F, AAGACTGCGTTCCTGCTCAAC; R, AAAGCCCTACAGCAACTGTCTG); *mHO-1* (F, AGGTGTCCAGAGAAGGCTTTAAG; R, TGCAGCTCCTCA GGAAGTAG); *mNrf2* (F, AAGCACAGCCAGCACTTCTC; R, GGCTTTTGTATGACCAAGGACTCAC); *mKeap1* (F, TCGAAGGCATCCACCCTAAG; R, CTCGAACACGCT-

GTC AATCT); *COX-2* (F, ATGCTGACTATGGCTACAAAAGC; R, TCGGGCAATCA TCAGGCAC); *NF- κB* (F, AACAGAGAGGATTTTCGTTCCG; R, TTTGACCTGAGGGTAAGACTTCT); *m β -actin* (F, ACTCCTATGTGGGTGACGAGG; R, CACACGCAGCTCATTGTAGAAG); and *GAPDH* (F, GCACCGTCAAGGCTGAGAAC; R, TGGTGAAGACGCCAGTGGA). All samples were analyzed in at least 3 different experiments performed in triplicates for the expression of each gene of interest.

Knockdown of Bach1 by Specific RNA Interference

To knockdown the expression of Bach1, cells were transfected with small interfering RNA (siRNA) using the siPORTTM Amine Transfection Agent (AM4503, Ambion). The siRNA was diluted to 2 and 10 nM in the OPT medium (HyClone), and then incubated to allow formation of siRNA complexes with 2 μL of the amine transfection agent. The siRNA complex was then added to each well of a 6-well plate to which approximately 2.5×10^5 cells in 1.1 mL of normal culture medium had been added. About 0.6 mL of fresh medium was added 12 h later and further incubated for up to 36 h before analyzing Bach1 and HO-1 expression [26].

Measurement of Intracellular ROS

Production of ROS was examined by the fluorescent staining with dihydroethidium (DHE). Once ROS, particularly superoxide anion, were generated in the cells, they enabled a chemical reaction with DHE to emit a red fluorescence under microscopy. The experimental cells were incubated with 10 μM of DHE in PBS for 30 min at 37°C. After 3 washes with PBS, the intracellular ROS were visualized under fluorescence microscope at the excitation and emission wavelengths of 480 and 530 nm, respectively. The collected images were quantified by the relative fluorescence intensity that was measured by the Image-Pro Plus 7.0 software to calculate the integrated optical density in the indicated cell area, and the significant difference of the results obtained was analyzed using Origin software.

Statistical Analysis

The data obtained from 3 individual experiments performed in triplicate for each sample are presented as means $\pm$ SD. The resulting statistical significance was calculated by a Student *t* test and *p* values of <0.05 for all relative experiments were considered as significant.

Results

AKBA Affects UVA-Induced Morphology, Viability, and ROS Levels

We first examined whether UVA irradiation affects cell morphology, cell viability, and the production of ROS in keratinocytes under current conditions. Twelve hours after UVA irradiation (80–400 kJ/m^2), fluorescence microscopy revealed morphological changes at doses >160 kJ/m^2 , with the cell shape being either shriveled or rounded (Fig. 1a). Reduction in cell viability ranged from 10 to 63% following 80–400 kJ/m^2 UVA irradiation (Fig. 1b). At 24 and 48 h after UVA irradiation, similar results were

Fig. 1. The effect of AKBA on UVA-induced cytotoxicity in human keratinocytes HaCaT. Cells (70–80% confluent) were sham (0 kJ/m²) and UVA (80–400 kJ/m²) irradiated, then incubated at 37°C in a humidified incubator (95% air, 5% CO₂). **a** Cell morphology was assessed under a fluorescence microscope 12 h after UVA irradiation. **b** Cell survival rate was evaluated by MTT assays 12 h after UVA irradiation. **c** Cellular ROS levels were detected by DHE staining immediately after UVA irradiation. **d** Cells were preincubated with 0.125–10 μM AKBA for 12 h and cell viability was mea-

sured by MTT. **e** Cells were pretreated with AKBA (0.125–2.5 μM), washed, and exposed to 160 kJ/m² of UVA irradiation in the presence of 1% FBS medium, then cultured for a further 12 h after irradiation, followed by cell viability determination using MTT. Values are expressed as relative percentage to the sham control, which was set to 1 (= 100%). Data are presented as means ± SD (*n* = 3). A Student *t* test was used for statistical significance with * *p* < 0.05; ** *p* < 0.01. Results are representative of 3 independent experiments.

seen (data not shown). As a result, the time course of 12 h after UVA irradiation was selected for most of the following experiments unless otherwise stated. DHE staining revealed a dose-dependent increase in the levels of ROS production from 80 to 400 kJ/m² (Fig. 1c). Cell viability loss was 20% and no morphology changes were observed at the UVA irradiation dose of 160 kJ/m²; therefore, this UVA dose was used throughout the experiments unless otherwise stated, which is comparable with previous studies [21, 27].

AKBA has anticancer properties that may involve inhibition of NF- κ B- and COX-2- mediated signaling pathways as demonstrated for intestinal carcinogenesis [7–10]. To evaluate whether AKBA has a protective effect against UVA-induced damages, HaCaT cells were pretreated with AKBA for 12 h prior to UVA irradiation. No significant changes in cell viability could be observed after 12 h of incubation with 0.125–5.0 μ M AKBA alone (Fig. 1d). Twelve hours after UVA irradiation at 160 kJ/m², the cell viability was decreased by 22%, but 0.5- μ M AKBA pretreatment significantly reduced the UVA-irradiated cell viability loss to 11% ($p < 0.05$, when compared to the vehicle-treated cells) (Fig. 1e). AKBA also caused a significant reduction in cell viability loss induced by hydrogen peroxide (H₂O₂) (online supplementary Figure Sfig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000452744). These data showed that AKBA prevents UVA-induced increase of cellular ROS levels and cell viability loss.

Chemoprevention of AKBA against UVA-Induced Cell Damage

To determine whether AKBA has a chemopreventive effect against UVA-induced oxidative damage, we pretreated HaCaT cells with AKBA followed by DHE staining to measure changes in intracellular levels of ROS (mostly O₂[•]) derived in cells irradiated with UVA (160 kJ/m²). A significant increase in fluorescent DHE staining occurred (Fig. 2a), indicating high intracellular ROS levels produced in UVA-irradiated HaCaT cells. Interestingly, the increased UVA-derived ROS was significantly blocked by AKBA ($p < 0.05$); the relative intensity of intracellular ROS declined from ~125% (UVA alone) to ~108% (UVA + AKBA) by 17% (Column [Co]: b–f) in a dose-dependent fashion (Fig. 2a).

UVA irradiation induced lipid peroxidation which caused a significant damage of cellular molecules, including antioxidant enzymes [28]. The lipid peroxidation product MDA was promoted by UVA-derived oxidative stress and pretreatment with AKBA caused a significant

reduction of MDA formation from 4.3-fold to 1.7-fold (* $p < 0.05$, ** $p < 0.01$) after UVA irradiation (Fig. 2b). UVA irradiation decreased SOD activity to 60%, while AKBA pretreatment (0.25–2.5 μ M) significantly increased the release of SOD in HaCaT cells with up to 30% of recovery ($p < 0.05$) (Fig. 2c) in a concentration-dependent manner.

Activation of Nrf2 by AKBA Promotes the Expression of Antioxidant Enzymes

We next investigated whether the observed cytoprotection of AKBA against UVA-induced oxidative stress occurs through activation of Nrf2 and its target antioxidant enzymes. First, a dose-dependent increase in Nrf2 protein expression induced by AKBA in HaCaT cells was detected. Concomitantly, the AKBA-induced Nrf2 increase was accompanied by a dose-dependent decrease in Bach1 protein levels (Fig. 3a). Notably, treatment of HaCaT cells with AKBA alone induces not only an up-regulation of Nrf2 (3.25-fold) (online suppl. Sfig. 2), NQO1 (3-fold), and HO-1 (1.4-fold), but also a down-regulation of Bach1 (0.66-fold) in a dose-dependent manner; however, no significant change in GSTP1 levels were observed under AKBA treatment (Fig. 3a). UVA irradiation significantly stimulated expression of Nrf2 (~1.5-fold) as well as its target HO-1 (~1.4-fold) on the protein level, while NQO1 (~1.1-fold) did not increase significantly (Fig. 3b and online suppl. Sfig. 2). In addition, irradiation significantly caused a decrease of the level of Bach1 to 0.82-fold when compared to sham-irradiated control (Fig. 3b). In contrast, AKBA pretreatment of UVA-irradiated cells led to a further increase in the protein levels of Nrf2, HO-1, and NQO1 to 3.5-fold, 3.1-fold, and 3.6-fold, respectively, and with a marked further decrease in Bach1 level by 0.56-fold, in a concentration-dependent way, as compared to UVA irradiation alone. However, no effect of AKBA on GSTP1 level in UVA-irradiated HaCaT cells was observed. In addition, gene transcription analysis also showed a significant increase of Nrf2 and HO-1 at 6 h after UVA irradiation (online suppl. Sfig. 1b).

The Effect of AKBA on the Expression of HO-1 or Nrf2, and Cell Viability in Bach1 Depletion or in Keap1^{-/-} Cells following UVA Irradiation

Previously we described that in HaCaT cells, HO-1 expression was increased with Bach1 RNA interference, but downregulated by Nrf2 RNA interference [19, 21]. According to these findings, we speculated that AKBA may modulate UVA-induced cellular damage via regulation of

Fig. 2. Protective effect of AKBA on UVA-induced cell damage. **a** HaCaT cells were preincubated with 0.25–10 μ M of AKBA for 12 h, washed with PBS, and exposed to UVA irradiation (160 kJ/m²); intracellular ROS levels were determined immediately following irradiation: A = sham, B = UVA, C = AKBA 0.25 μ M + UVA, D = AKBA 0.5 μ M + UVA, E = AKBA 1.0 μ M + UVA, and F = AKBA 2.5 μ M + UVA. Cells were treated with AKBA (0.25–2.5 μ M)

for 12 h before exposure to UVA (160 kJ/m²); MDA production (**b**) and SOD activities (**c**) were determined 2.5 h after irradiation. Values are expressed as the relative percentage to sham control, which was set to 1 (= 100%). Data are presented as means $\pm$ SD ($n = 3$). A Student *t* test was used for statistical significance with * $p < 0.05$; ** $p < 0.01$. Results are representative of 3 independent experiments.

Fig. 3. AKBA modulates UVA irradiation-induced Nrf2 and HO-1 levels. AKBA affects levels and distribution of Nrf2, NQO1, HO-1, and Bach1 in UVA-irradiated HaCaT cells. **a** HaCaT cells were pretreated with AKBA (0.25–2.5 μ M) for 12 h. Nrf2, HO-1, NQO1, Bach1, and GSTP1 protein levels were analyzed by immunoblots and are shown as relative values with the control (no AKBA) set to 1. **b** Total protein was extracted 12 h following UVA irradiation (160 kJ/m²) with AKBA pretreatment, and protein levels were detected by immunoblot. **c** Effect of AKBA on HO-1 expression and cell viability in Bach1-silenced HaCaT cells: AKBA-treated (0.5 μ M) HaCaT cells were transfected with scramble siRNA (control) or Bach1 siRNA (siBach1) for 36 h using siPORTTM Amine Transfec-

tion Agent (Ambion), as described in Materials and Methods. Then, cells were irradiated with UVA. **d** WT and Keap1^{-/-} cells were pretreated with AKBA 0.5 μ M for 12 h, followed by irradiation with UVA. Expression levels of Nrf2 and HO-1 were detected by RT-PCR and cell viability was determined by MTT 6 and 12 h after UVA, respectively. Relative gene expression of HO-1 or Nrf2 is shown in the bar graph as a ratio of the normalized value relative to sham controls, which was set to 1 (= 100%). Data are presented as means $\pm$ SD ($n = 3$). A Student *t* test was used for statistical significance with * $p < 0.05$; ** $p < 0.01$. Results are representative of 3 independent experiments.

(For figure see next page.)

a

b

c

d

Fig. 4. AKBA modulates UVA-induced inflammatory response and NOX1 levels. AKBA reduces UVA-induced inflammatory factors *COX-2* and *NF-κB* in HaCaT cells: cells were treated with AKBA (0.25–2.5 μM) and irradiated with UVA (160 kJ/m²). RNA/cell lysates were collected 3 h (RNA) and 6 h (protein) after UVA irradiation, and were then used for qPCR (*COX-2*, *NF-κB*) (**a**) or for immunoblots using *COX-2* and p65RelA (*NF-κB*) antibodies (**b**). **c** AKBA suppresses the level of UVA irradiation-induced NOX1. HaCaT cells were irradiated with UVA (160 kJ/m²) and

incubated for up to 24 h. **d** Protein levels of NOX1 were determined by Western blotting. AKBA- and DPI-treated cells were irradiated by UVA and then incubated for 3 h. Total cellular protein was extracted and NOX1 detected by Western blotting. Signal intensities were determined by densitometry and values are expressed as the relative percentage to the sham control, which was set to 1. Data are presented as means ± SD (n = 3). Student's *t* test was used for statistical significance with * *p* < 0.05; ** *p* < 0.01. Results are representative of 3 independent experiments.

Bach1/Nrf2-mediated HO-1 expression. The effect of AKBA on HO-1 induction was examined in Bach1 knock-down HaCaT cells. Previously, RT-PCR analyses showed that RNAi silencing of Bach1 increased the expression of the HO-1 gene [19]. Here we found that HO-1 gene ex-

pression is further increased by 48% upon AKBA treatment in siBach1 cells compared to siBach1 cells (Fig. 3c); however, under these conditions, cell viability was not significantly increased. Also, qPCR analysis showed that HO-1 gene expression increased by 20% with 0.5 μM

AKBA in siBach1 cells after UVA irradiation compared to the sham irradiation ($p < 0.05$). Notably, UVA irradiation also caused an increase in HO-1 expression by 19% in AKBA-pretreated siBach1 cells, and cell viability was significantly increased by 12% ($p < 0.05$). Moreover, UVA-induced cell viability loss was recovered when treated with AKBA (10%) and siBach1 (15%), while in combination (AKBA + siBach1) a significant (25%) increase in cell viability was observed (Fig. 3c).

It was found that under UVA-induced oxidative stress, resveratrol enhanced the degradation of Keap1 and nuclear translocation of Nrf2, and helped cells to escape cellular stress in order to promote cellular survival [29]. Keap1-deficient or -depleted skin cells were shown to have increased expression of both Nrf2 and HO-1 (siKeap1) and are more resistant to UVA-mediated damage [22]. WT and Keap1-deficient skin fibroblasts cells were used to analyze how AKBA affects cellular response to UVA; in Keap1^{-/-} cells, we confirmed the increase of expression levels of HO-1 and Nrf2 (Fig. 3d). Interestingly, Nrf2 and HO-1 expression levels were further increased by 25% (Co: 14, 16) and 37% (Co: 13, 15), respectively, in Keap1^{-/-} cells after UVA irradiation with AKBA pretreatment as compared to UVA irradiation alone. Furthermore, AKBA treatment increased Nrf2 (Co: 7, 15) and HO-1 (Co: 8, 16) gene expression, respectively ($p < 0.05$). Under these conditions, cell viability was increased by 15% (Co: 7, 8). In Keap1^{-/-} cells (UVA + AKBA) compared to Keap1^{+/+} WT (UVA + AKBA), cell viability was increased by 19% (Co: 4–8) (Fig. 3d). These data indicate that AKBA acts similar as a Keap1 deficiency and may protect cells against UVA exposure via increase of Nrf2 and HO-1 expression levels.

AKBA Modulates UVA-Induced Inflammatory Mediators

To test whether AKBA has an anti-inflammatory effect against UVA-induced cell damage, changes in COX-2 and NF- κ B (p65RelA) mRNA (3 h) and protein levels (6 h) were determined. Gene analysis showed that COX-2 and NF- κ B were both induced 6 h after UVA irradiation, but the induction rates were significantly reduced by 33% (COX-2) and 43% NF- κ B, respectively, in UVA-irradiated HaCaT cells with AKBA pretreatment (Fig. 4a). Further, the UVA-stimulated increase of both proteins COX-2 and NF- κ B were significantly reduced upon AKBA pretreatment in a dose-dependent manner (Fig. 4b).

In order to investigate the effect of AKBA on NOX1, the major sources of ROS in keratinocytes, we determined the protein level of NOX1 after UVA irradiation. Data

showed that the peak time of NOX1 induction is 3 h after UVA irradiation under the conditions we used; DPI reduced the expression level of NOX1 and the inhibitory effects of DPI on NOX1 was used as positive control (Fig. 4c). AKBA (0.25–2.5 μ M) enabled a significant reduction of UVA-induced NOX1 expression from 2.3- to 1.4-fold ($p < 0.05$), in a concentration-dependent manner (Fig. 4d)

Discussion

The longer wavelength UVA (320–400 nm) radiation either causes direct damage or may enhance short wavelength UVB damaging effects to the skin [9, 26]. Besides direct damage to macromolecules such as nucleotides (i.e., DNA and RNA), lipids, and proteins, both UVA and UVB exert effects through triggering oxidative stress [1, 30]. In HaCaT cells (70–80% confluence) we observed that 160 kJ/m² of UVA irradiation caused a cell viability loss of 22%. Cell viability loss was obtained by UVA irradiation with 40–160 kJ/m² using the same UVA lamp setup, as described previously [24, 25].

A number of natural products from plants like phenolics and flavonoids [17], peanut sprout extracts [31], and afzelin [32], etc., have been reported as very useful chemoprevention against oxidative stress. Our present study provides evidence that the plant derivative AKBA is a promising regimen for chemoprevention through activation of the Nrf2-ARE-driven antioxidant pathway; researchers showed that AKBA suppresses tumorigenesis, inflammation, and neuroprotection [8, 33]. Further, we provided evidence that AKBA rescued UVA-induced cell viability loss through alleviating ROS in HaCaT cells. As a consequence of ROS generation by UVA irradiation, peroxidation products of polyunsaturated fatty acids are generated that yield a range of aldehydes and carbonyls, of which one such compound is MDA [14]. UVA-induced ROS and other free radicals are detoxified by the endogenous antioxidant SOD, which catalyzes the dismutation of superoxides (O₂⁻) into oxygen and H₂O₂, thereby protecting against oxidative damage [34]. AKBA boosts the induction of SOD expression to significantly high levels and consequently decreases the accumulation of MDA and ROS to mediate cytoprotection of UVA-irradiated HaCaT cells against oxidative stress.

The putative UVA-induced oxidative stress is thought to evoke translocation of Nrf2 into the cell nucleus, thus allowing upregulation of antioxidant and detoxification enzymes, such as HO-1, NQO1, and GST [20, 35]. These

enzymes are found to be crucial for the protection of cells from oxidative stress, as well as for preventing UVA-induced carcinogenesis [36]. For instance, Nrf2-HO-1 proteins are rapidly induced by UVA, H₂O₂ and many others stressors that lead to cytoprotection against oxidative stress [21, 36–38]. HO-1 may protect keratinocytes against UVA-mediated damage, but also protects cancer cells against chemotherapy via induction of these phase II enzymes [25, 39, 40].

Cotreatment of AKBA and UVA can further increase expression of Nrf2 which targets HO-1 and NQO1. AKBA promotes the nuclear accumulation of Nrf2 and reduced Bach1 expression, and thus contributes to the activation of their target genes NQO1 and HO-1. Collectively, AKBA prevents HaCaT cells from UVA-induced damage primarily by upregulating the Nrf2-ARE-driven antioxidant and detoxification enzymes with Bach1 repression, and therefore leads to expression of HO-1.

Irradiation of human skin cells with UVA causes accumulation of ROS and induces expression of inflammatory mediators such as COX-2. UVA-stimulated expression of COX-2 and NF- κ B are significantly inhibited by AKBA at a concentration of 2.5 μ M. Interestingly, AKBA prevents inflammatory factors arising from both small intestinal and colonic polyps [8, 34].

We also found that AKBA has an antioxidant property that inhibits NOX1 activity, a major mediator of UVA-derived ROS generation in skin keratinocytes. In addition, AKBA downregulates expression of Bach1 and further promotes expression of HO-1 upon knockdown of Bach1. A similar upregulation of HO-1 and Nrf2 has been described in Keap1^{-/-} cells after exposure to UVA irradiation [22]. Our study indicates that AKBA may further reduce the oxidative stress induced by UVA-irradiation.

In conclusion, our present study reveals that the small molecule AKBA upregulates expression of Nrf2-directed

antioxidant and detoxification enzymes (i.e., HO-1 and NQO1) and downregulates the inflammatory responses mediated by COX-2 and NF- κ B; simultaneous regulation of these 2 processes reduces NOX1, thus providing cytoprotection to human keratinocytes against UVA-induced oxidative stress. Overall, AKBA could be considered as a potential therapeutic agent to target the Nrf2-ARE-driven gene regulatory pathway against UVA-induced photodamage and cancer.

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Statement of Ethics

The research did not involve human participants and/or animals. The authors have no ethical conflict to disclose.

Disclosure Statement

The authors have declared no conflicting interests.

Author Contributions

Shiying Yang, Bin Zhou, Xu Wei, Fangfang Xue, Chunxiang Bian, and Yiguo Zhang designed and performed the study and analyzed the data. Muhammad Farrukh Nisar and Li Yang contributed to data collection and analysis. Julia Li Zhong and Jörg W. Bartsch designed and wrote the paper. All authors have read and approved the final paper.

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