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Original Contribution

Baicalein protects Human melanocytes from H_2O_2-induced apoptosis via inhibiting mitochondria-dependent caspase activation and the p38 MAPK pathway

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ABSTRACT

The removal of H_2O_2 by antioxidants has been proven to be beneficial to patients with vitiligo. Baicalein (5,6,7-trihydroxyflavone; BE) has antioxidant activity and has been used in vitiligo therapy in Chinese traditional medicine. In this study, we investigated the potential protective effect and mechanisms of BE against H_2O_2-induced apoptosis in human melanocytes. Melanocytes from the PIG1 cell line were pretreated with different concentrations of BE for 1 h, followed by exposure to 1.0 mM H_2O_2 for 24 h. Cell apoptosis, reactive oxygen species levels, and mitochondrial membrane potentials were evaluated by flow cytometry, and cell viability was determined by an MTT assay. The expressions of Bax, Bcl-2, caspase-3, total and phosphorylated ERKs, and p38 MAPK were assayed by Western blot to investigate the possible molecular mechanisms. Our results showed that BE significantly inhibited H_2O_2-induced apoptosis, intracellular reactive oxygen species generation, and changes in the mitochondrial membrane potential. It also reduced the Bax/Bcl-2 ratio, the release of cytochrome *c*, the activation of caspase-3, and the phosphorylation of p38 MAPK in a concentration-dependent manner. The results demonstrate for the first time that BE exerts a cytoprotective role in H_2O_2-induced apoptosis by inhibiting the mitochondria-dependent caspase activation and p38 MAPK pathway.

Introduction

Vitiligo is an acquired and progressive depigmentation disorder for which the etiology and pathogenesis are still unclear. Studies suggest that oxidative stress caused by reactive oxygen species (ROS) plays a major role in the loss of melanocytes. H_2O_2, which is a main source of ROS, has been found to accumulate in the epidermis of acute vitiligo patients at a concentration of approximately 10^{-3} M [1–3]. The increase in H_2O_2 mainly occurs at the onset and during the progression phase, possibly contributing to the melanocyte damage characteristic of vitiligo [4,5]. Based on research by Schallreuter's group [6], the degeneration of melanocytes is commonly considered to be a result of H_2O_2 cytotoxicity. However, the mechanism by which H_2O_2 induces apoptosis in melanocytes is still under investigation.

The mitochondrial and death receptor pathways are two major apoptotic pathways in cells [7,8]. Studies have revealed that mitochondria contain the major source of oxidative stress and play crucial roles in cell apoptosis in several diseases. Mitochondrial dysfunction leads to the loss of the mitochondrial transmembrane potential and the release of cytochrome *c*, which causes caspase activation and cell apoptosis [9]. Several groups have reported that impaired mitochondrial ultrastructure and function, as well as intense lipid peroxidation, were present in the perilesional skin and blood of vitiligo patients [10,11]. Activation of the mitogen-activated protein kinase (MAPK) family has been implicated in cell death induced by oxidative stress [12,13]. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun N-terminal kinase (JNK) [14]; the associated signaling cascades play key roles in a variety of cellular responses, such as cell proliferation, differentiation, and apoptosis [15]. Evidence suggests that constant accumulation of H_2O_2 leads to a cascade of impaired signals in active vitiligo [16–20]. H_2O_2-mediated oxidative stress is a putative mediator of

Abbreviations: Bax, B cell lymphoma 2-associated X protein; Bcl-2, B cell lymphoma 2; BE, baicalein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MTT, 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer solution; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; TBS, Tris buffer solution

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melanocyte death in vitiligo, and removal of H_2O_2 by antioxidants has been proven to be beneficial to patients with vitiligo. Therefore, we hypothesized that agents aimed at preventing or attenuating H_2O_2-induced cell damage and mitochondrial dysfunction might be a possible approach for inhibiting the degeneration of melanocytes in vitiligo.

Baicalein is a major flavonoid in *Scutellaria baicalensis*, which is an herb that has been used extensively in traditional Asian medicine [21]. It is reported that BE exhibits a variety of biological effects, including anti-inflammatory, anticytotoxicity, antiviral, and antitumor actions [22,23]. In addition, BE has strong antioxidant activity against ROS and inhibits lipid peroxidation in mitochondria [24,25]. Many studies have shown that BE inhibits H_2O_2-induced oxidative damage and apoptosis in several types of cells [26–28]. Accumulating evidence suggests that BE can act selectively within MAPK signaling cascades [26,28]. However, the role of MAPK in the action of BE on human melanocytes and the ability of BE to protect these cells against H_2O_2-induced oxidative damage and apoptosis are still unknown.

Therefore, the purpose of our study was to investigate whether BE protects human melanocytes from H_2O_2-induced oxidative stress and apoptosis and to elucidate the underlying molecular mechanism. In the present study, we used H_2O_2-induced oxidative stress in the normal human melanocyte cell line PIG1 as an in vitro model. This study sheds new light on the free radical scavenging as well as antiapoptosis properties of BE, with reference to H_2O_2-induced oxidative stress and cytotoxicity.

Materials and methods

Chemicals

The following reagents were obtained from the indicated commercial sources. Baicalein, hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), annexin V-FITC apoptosis detection kit, sodium deoxycolate, 2′,7′-dichlorofluorescein-diacetate (DCF-DA), a cocktail of protease inhibitors, Hoechst 33258, rhodamine 123, SB203580 (p38 MAPK inhibitor), U0126 (ERK inhibitor), and Z-VAD-FMK (pan-caspase inhibitor) were obtained from Sigma-Aldrich (MO, USA). Rabbit anti-Bax, anti-Bcl-2, anti-cytochrome *c*, anti-total caspase-3, and anti-cleaved caspase-3 were obtained from Epitomics (Epitomics, CA, USA). Rabbit anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK, anti-phospho-ERK, and anti-cytochrome oxidase subunit IV (COX4) were obtained from Cell Signaling (Cell Signaling Technology, MA, USA). A monoclonal mouse anti-actin antibody was obtained from Biomeda Corp. (CA, USA). A bicinchoninic acid (BCA) protein assay kit and cell lysis buffer were obtained from Thermo Fisher Scientific Inc. (IL, USA). Lactate dehydrogenase (LDH) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Cell culture and treatment

Immortalized human epidermal melanocyte cells from cell line PIG1 (a gift from Dr. Caroline Le Poole, Loyola University Chicago, Maywood, IL, USA) were cultured in Medium 254 (Cascade Biologics/Invitrogen, Portland, OR, USA) supplemented with human melanocyte growth supplement (Cascade Biologics/Invitrogen), 5% fetal bovine serum (Invitrogen, CA, USA), and penicillin-streptomycin antibiotic mix (Invitrogen) at 37 °C in the presence of 5% CO_2. Baicalein was freshly prepared as a stock solution in DMSO and diluted with Medium 254 supplements (0.1% (v/v) DMSO). DMSO was present at equal concentrations (0.03%) in all groups. H_2O_2 was freshly prepared from a 30% stock solution prior to each experiment.

Cell fractionation

Cytoplasmic and mitochondrial fractions were isolated by differential centrifugation using the mitochondrial fractionation kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the melanocytes were treated with BE and/or H_2O_2 in the indicated time points. The cells were harvested and washed once with PBS. Then the cells were resuspended for 15 min in ice-cold cytosolic buffer (Active Motif) and homogenized on ice using 30 strokes with the homogenizer. The homogenate was passed 20 times through a 22-gage needle to lyse cells. The nuclear fraction was recovered by centrifugation at 800*g* for 20 min at 4 °C. The "low-speed" pellet was lysed with lysis buffer on ice for 30 min. The "low-speed" supernatant was centrifuged at 10,000 *g* for 20 min at 4 °C to obtain the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). The mitochondrial fraction was further lysed in the mitochondria buffer (Active Motif) and incubated on ice for 15 min. After fractionation, the protein concentration in each fraction was measured. Western blot analysis of each fraction was performed.

Immunoblotting

After treatment, the cells were washed once with PBS and then lysed using ice-cold RIPA buffer with a protease inhibitor cocktail. The cell lysates were centrifuged at 12,000*g* for 25 min at 4 °C. The protein concentrations were determined by the BCA method using bovine serum albumin (BSA) as the standard. The proteins were separated by 10–15% SDS-PAGE and transferred to a polyvinylidine difluoride (PVDF) membrane. The membrane was blocked with 5% (v/v) nonfat dry milk in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) at room temperature (RT) for 2 h, followed by incubation overnight at 4 °C in 1:1000 dilutions of Bcl-2, Bax, cytochrome *c*, total caspase-3, cleaved caspase-3, p38 MAPK, phospho-p38 MAPK, ERK, and phospho-ERK primary antibodies. The membrane was washed three times with TBST for 5 min each. Next, the membrane was incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at RT for another 2 h and washed again three times in TBST buffer. The membrane was incubated with ECL substrate solution for 5 min according to the manufacturer's instructions and visualized with autoradiography film. The imaging program Quantity One (Bio-Rad) was used for quantification.

Cell viability analysis

Cell viability was monitored using an MTT assay according to the manufacturer's protocol. In brief, PIG1 cells were initially plated at a density of 1×10^4 cells per well in 96-well plates for 24 h. The cells were then preincubated with different concentrations of BE for 1 h before exposure to H_2O_2 (100 μL, 1.0 mM) for 24 h at 37 °C. Then, MTT (10 μL, 0.5 g/L) was added to each culture well and incubated for 4 h at 37 °C. The medium was pipetted out from each well, and 150 μL DMSO was added to dissolve the MTT-formazan crystals produced by the viable cells. The absorbance at 490 nm was measured with a microplate reader (Bio-Rad, CA, USA). The morphology of the cells was assessed by phase contrast using an inverted microscope (Nikon, TE-2000U, Japan). All experiments were performed in triplicate, and the results for the absorbance measured in treated cells were calculated as percentages of the absorbance in untreated control cells.