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Honokiol attenuates oxidative stress-induced cytotoxicity in human keratinocytes *via* activating AMPK signaling

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ABSTRACT

Objective: To investigate the effect of honokiol on oxidative damage in HaCaT human keratinocytes.

Methods: HaCaT cells were exposed to hydrogen peroxide (H_2O_2), following pretreatment with various concentrations of honokiol. The alleviating effects of honokiol on HaCaT cell viability and cell death, reactive oxygen species (ROS) production, DNA damage, mitochondrial dynamics, and inhibition of adenosine triphoaphate production against H_2O_2 were investigated. Western blotting analysis was used to analyze the expression levels of specific proteins.

Results: Honokiol suppressed H_2O_2-induced cytotoxicity and DNA damage by blocking abnormal ROS accumulation. Honokiol also prevented apoptosis by inhibiting loss of mitochondrial membrane potential and release of cytochrome c from the mitochondria into the cytosol, decreasing the Bax/Bcl-2 ratio, and reducing the activity of caspase-3 in H_2O_2-stimulated HaCaT cells. In addition, honokiol attenuated H_2O_2-induced reduction of adenosine triphosphate content, and activation of AMP-activated protein kinase (AMPK) was markedly promoted by honokiol in H_2O_2-stimulated cells. Importantly, the anti-apoptosis and anti-proliferative activity of honokiol against H_2O_2 was further enhanced by adding an activator of AMPK, indicating that honokiol activated AMPK in HaCaT keratinocytes to protect against oxidative damage.

Conclusions: The present results indicate that honokiol may be useful as a potential therapeutic agent against various oxidative stress-related skin diseases.

KEYWORDS: Honokiol; ROS; DNA damage; Apoptosis; AMPK

1. Introduction

Aerobic organisms like humans use oxygen as an electron acceptor during oxidative phosphorylation within mitochondria to create molecules with high energy needed for the cells to survive. This means that mitochondria can serve as the largest source of free oxygen radicals, such as reactive oxygen species (ROS), which play a crucial role in the processing of various biochemical signals within the cell[1,2]. Under normal physiological conditions, ROS act properly as intracellular signal transduction molecules in the cell, but the disordered increase in ROS synthesis due to the disturbances in pro-oxidant and antioxidant systems correlates with the occurrence of diverse diseases[3,4]. Human skin is a sensitive organ to external stimuli because it serves as a defense against harmful external environments. Keratinocytes are the major cellular component of the epidermis and are very vulnerable to oxidative damage, which can lead to serious skin-related diseases[5,6]. There is increasing evidence that excessive accumulation of ROS during continuous exposure to oxidative stress in most cells, including keratinocytes, causes oxidative modifications to nucleic acids, proteins, lipids, and other small intracellular molecules, eventually leading to DNA damage and cell death[6,7]. Therefore, the discovery of new antioxidants and research on their mechanisms have been recognized as a strategy for treating oxidative stress-mediated skin injuries.

Honokiol is a natural phenolic phytochemical and one of the main bioactive components widely contained in the seed cones and barks of the *Magnolia* species, which are traditionally used to treat a variety of diseases in Asian regions, including Korea[8,9]. According to many previous studies, honokiol has been found to have diverse biological activities besides cytotoxicity of cancer cells, including antiviral, antibacterial, anti-inflammatory, antioxidant, cardioprotective, liver protective and neuroprotective effects[10-14].

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These observations indicate that this compound can act as an anti-tumor as well as cytoprotective molecule. Recently, several molecular targets of honokiol have been identified, including sirtuin-3 (Sirt3) and AMP-activated protein kinase (AMPK)[15-18]. Activation of these molecules plays a key role in cell survival, proliferation, metabolism, differentiation, and apoptosis. In particular, AMPK, a regulator of energy metabolism, monitors cellular energy status to directly stimulate mitochondrial energy production and enhance mitochondrial biogenesis[19,20]. Several previous studies indicated that honokiol enhances mitochondrial function and reduces lipid peroxidation and ROS accumulation through activation of Sirt3, and AMPK acts as a downstream signaling modulator of Sirt3 in this process[21-25]. Therefore, these results imply that the potential antioxidant effect of honokiol is achieved through Sirt3-mediated AMPK activation. However, the beneficial effects of honokiol on skin cells under oxidative stress and the potential mechanisms are unknown. Therefore, in the present study, the protective effect of honokiol against the cytotoxicity induced by oxidative stress using hydrogen peroxide (H_2O_2) in human immortalized keratinocytes (HaCaT) was evaluated. The involvement of the AMPK signaling in the honokiol-mediated antioxidant mechanism was also investigated.

2. Materials and methods

2.1. Cell culture and honokiol treatment

HaCaT cells purchased from the American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and antibiotics mixture (WelGENE Inc., Gyeongsan, Republic of Korea) using a water-saturated humidified incubator at 5% CO_2 and 37 ℃. Honokiol obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) was dissolved in dimethyl sulfoxide and then diluted in Dulbecco's modified Eagle medium prior to use in the experiment to obtain the appropriate concentration. The concentration of H_2O_2 for the establishment of oxidative mimic conditions in HaCaT cells was selected to be 500 μM with a survival rate of about 60%.

2.2. Cell viability assay

To assess cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, HaCaT cells were treated with different concentrations of honokiol for 24 h, or pre-treated with honokiol (10 μM), *N*-acetyl-*L*-cysteine (10 mM, NAC, Sigma-Aldrich Chemical Co.) or 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; Sigma-Aldrich Chemical Co.) for 1 h, and then incubated with or without H_2O_2 (500 μM, Sigma-Aldrich Chemical Co.). After 24 h, the MTT solution (Sigma-Aldrich Chemical Co.) was added to the culture plate at a final concentration of 0.5 mg/mL and reacted at 37 ℃ for 3 h. The culture supernatant was then carefully discarded, and the formed formazan crystals were immediately dissolved using dimethyl sulfoxide. As previously described[26], the optical density of the formazan crystals formed in the cells was measured at 570 nm absorbance using an ELISA reader (Dynatech Laboratories, Chantilly, VA, USA), and the optical density in untreated control cells was considered 100% alive.

2.3. Nuclear staining assay

Using 4,6-diamidino-2-phenylindole (DAPI) staining, the changes in nuclear morphology were examined. Briefly, HaCaT cells treated with H_2O_2 in the presence or absence of honokiol or NAC were harvested and washed with phosphate-buffered saline (PBS). The cells were fixed with 3.7% paraformaldehyde (Sigma-Aldrich Chemical Co.) for 10 min at room temperature and then stained with DAPI solution (2.5 μg/mL, Sigma-Aldrich Chemical Co.) for 10 min at room temperature. After washing with PBS again, DAPI-stained nuclei were observed through a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.4. Annexin Ⅴ staining

The evaluation of apoptosis was performed using an annexin Ⅴ-fluorescein isothiocyanate (FITC) apoptosis detection kit purchased from R&D Systems Inc. (Minneapolis, MN, USA) according to manufacturer's instructions. In brief, the collected HaCaT cells were washed with PBS and fixed in 75% ethanol at 4 ℃ for 30 min. The cells were then stained with annexin Ⅴ-FITC and propidium iodide (PI) at room temperature for 20 min. The fluorescence intensities of the cells were quantified using a flow cytometer (Becton Dickinson, San Jose, CA, USA) to evaluate the degree of apoptosis induction, as previously described[27].

2.5. Determination of ROS production

To measure the amount of ROS generated in cells, HaCaT cells were pre-treated with or without honokiol or NAC for 1 h before another 1 h culture in the absence or presence of H_2O_2. After washing with PBS, the cells were lysed with PBS containing 1% Triton X-100 at 37 ℃ for 10 min and then stained with 2',7'-dichlorofluorescein diacetate (10 μM, DCF-DA, Molecular Probes, Eugene, OR, USA) at room temperature for 30 min. Intracellular ROS production was immediately recorded at 515 nm absorbance using a flow cytometer as previously described[28]. The changes in ROS were also analyzed by fluorescence microscopy. To this end, cells cultured in glass coverslips were exposed to H_2O_2 in the absence or presence of honokiol or NAC for 1 h. The cells were also stained with DCF-DA (10 μM) at room temperature for 30 min and fluorescent images were immediately captured under a fluorescence microscope.

2.6. Comet assay

After stimulating cells with H_2O_2 alone or with honokiol, DNA damage was assessed by a comet assay as described by Aristizabal-Pachon and Castillo[29]. Briefly, the cells washed with PBS were suspended in low melting agarose at 37 ℃ and immediately spread on microscope slides coated with normal melting agarose. After solidifying the agarose, the slides were covered with low melting

agarose and immersed in the lysis solution at 4 ℃ for 1 h. The slides were incubated for 30 min in a gel electrophoresis device and electrophoresed for 20 min. After electrophoresis, the slides were washed twice with neutralizing buffer and then stained with PI solution (20 μg/mL). The resulting nuclear images were observed using a fluorescence microscope.

2.7. Determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentration

The levels of 8-OHdG were calculated using the commercially available 8-OHdG ELISA Kit (BIOXYTECH® OXIS International Inc., Foster City, CA, USA) according to the instructions. In brief, the cellular DNA of HaCaT cells exposed to H_2O_2 with or without honokiol or NAC was extracted using the Genomic DNA purification kit (Promega Corporation, Madison, WI, USA). Then, DNA was sequentially digested using DNase Ⅰ and alkaline phosphatase at 37 ℃ for 1 h. The amount of 8-OHdG was quantified using an ELISA plate reader at 450 nm based on the manufacturer's instruction. Subsequently, the concentration of 8-OHdG for each sample was quantified from the standard curve following manufacturer's recommendations.

2.8. Western blotting analysis

HaCaT cells treated with H_2O_2 in the presence or absence of honokiol or NAC or AICAR were harvested and the collected cells were lysed with lysis buffer as previously described[30]. The cytosolic and mitochondrial proteins were extracted using a mitochondrial fractionation kit obtained from Active Motif, Inc. (Carlsbad, CA, USA) according to the manufacturer's procedure. The same amounts of protein were separated by electrophoresis using sodium-dodecyl sulfate-polyacrylamide gel, and then the protein in the gel was transferred to polyvinylidene difluoride membranes (Merck Millipore, Bedford, MA, USA). Subsequently, the membranes were incubated with primary antibodies at 4 ℃ overnight. Anti-histone variant H2AX (γH2AX, D17A3), phospho (p)-γH2AX (Ser139, 20E3), p-AMPK (2535) and p-ACC (3661) were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). The primary antibodies against cytochrome c (sc-7159), cytochrome oxidase subunit 4 (COX Ⅳ, sc-23986), AMPK (sc-74461), acetyl-coenzyme A-carboxylase (ACC, sc-137104), poly(ADP-ribose) polymerase (PARP, sc-8007), caspase-3 (sc-7272), Bax (sc-7480), Bcl-2 (sc-7382) and actin (sc-7210) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). After washing with PBS, the membranes were reacted with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science, Arlington Heights, IL, USA) for 2 h at room temperature. Protein bands were detected using an enhanced chemiluminescence reagent (Amersham Life Science).

2.9. Detection of mitochondrial membrane potential (MMP, $\Delta\psi m$)

MMP was measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1). Briefly, the collected cells were incubated with JC-1 (10 μM, Sigma-Aldrich Chemical Co.) at 37 ℃ for 20 min. The cells were washed with PBS and analyzed by a flow cytometer. JC-1 monomer and JC-1 aggregate fluorescence were quantified using emission filters of 535 nm and 595 nm, respectively, according to the manufacturer's protocol. Cells that lost MMP are indicated using the percentage of JC-1 monomer.

2.10. Detection of adenosine triphoaphate (ATP) levels

Using a firefly luciferase-based ATP bioluminescence assay kit (Roche Applied Science, Indianapolis, IN, USA), the levels of intracellular ATP were measured. In brief, the cells cultured at various conditions were lysed with the lysis buffer and the supernatant was collected according to the instructions. The same amount of supernatant and luciferase reagent provided in the kit were mixed. The activity of firefly luciferase was immediately measured using a luminometer (Turner Designs, Inc. San Jose, CA, USA) and the levels of ATP were determined according to the ATP standard curve. To compare ATP concentrations between treatment groups, ATP levels were expressed as nmole per mg cell protein.

2.11. Statistical analysis

All results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's *post-hoc* test using GraphPad Prism software (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Honokiol suppresses cytotoxicity induced by H_2O_2 in HaCaT cells

As shown in Figure 1A, there was no significant difference in viability in HaCaT cells treated with honokiol no more than 10 μM compared with the control group. Therefore, to investigate the cytoprotective effect of honokiol against cytotoxicity by H_2O_2, a 10 μM concentration of honokiol was chosen. The results demonstrated that honokiol significantly inhibited the decrease in HaCaT cell viability induced by H_2O_2 (Figure 1B). In addition, it was also found that NAC, a well-established ROS scavenger, had a significant protective effect on H_2O_2-induced cytotoxicity compared with the control (Figure 1B).

3.2. Honokiol inhibits apoptosis induced by H_2O_2 in HaCaT cells

To validate the ability of honokiol to prevent H_2O_2-induced apoptosis, DAPI staining and annexin Ⅴ-FITC/PI assays were performed. As shown in the representative images in Figure 2A, apoptotic nuclei observed by DAPI staining were clearly increased in H_2O_2-treated HaCaT cells, but pretreatment with honokiol or NAC markedly reduced these morphological changes. The results of annexin Ⅴ/PI staining

also showed that H_2O_2 triggered apoptosis (Figure 2B). However, after pretreatment with NAC as well as honokiol, the percentage of apoptotic cells was significantly decreased.

3.3. Honokiol reduces ROS generation induced by H_2O_2 in HaCaT cells

As shown in Figure 3A and B, the production of ROS in HaCaT cells treated with H_2O_2 for 1 h increased more than 5 times compared with the control group. However, pretreatment with honokiol significantly attenuated the effect of H_2O_2 on ROS production, and NAC also markedly eliminated the accumulation of ROS (Figure 3A and B). Similarly, honokiol and NAC protected against H_2O_2-induced DCF fluorescence in the cells (Figure 3C), demonstrating that honokiol has potent ROS scavenging activity.

3.4. Honokiol protects against DNA damage induced by H_2O_2 in HaCaT cells

To examine whether honokiol can prevent H_2O_2-induced DNA damage, an alkaline comet assay was performed. As shown in Figure 4A, no apparent comet tail moment (DNA migration) was observed in HaCaT cells treated with honokiol alone. However, in H_2O_2-treated cells, markedly increased DNA migration, indicating DNA damage, was observed, which was clearly reduced in the presence of honokiol and NAC. Additionally, the DNA damage blocking effect

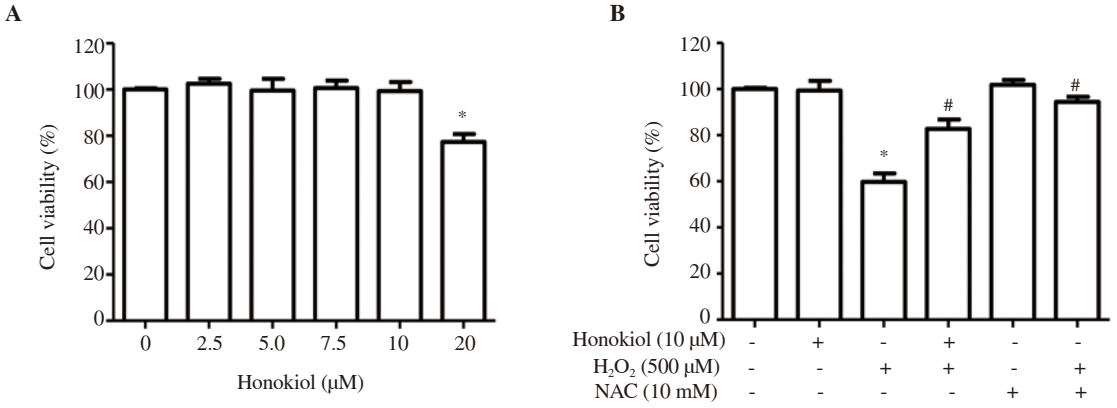

Figure 1. Protective effect of honokiol on H_2O_2-induced cytotoxicity in HaCaT cells. HaCaT cells were stimulated with various concentrations of honokiol (A) for 24 h or pre-treated with or without honokiol (10 μM) or NAC (10 mM) for 1 h and then cultured in the presence or absence of H_2O_2 (500 μM) for 24 h (B). The cell viability was determined by MTT assay. The results are expressed as mean ± SD of three independent experiments ($^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the H_2O_2-treated group). NAC: *N*-acetyl-*L*-cysteine.

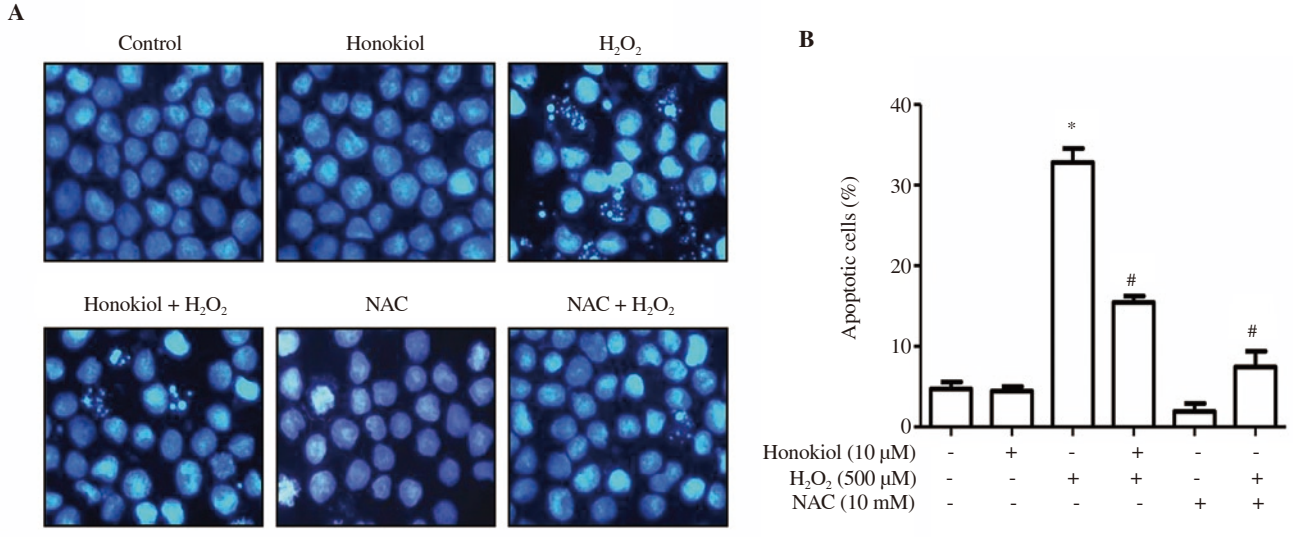

Figure 2. Suppression of H_2O_2-induced apoptosis by honokiol in HaCaT cells. HaCaT cells were pre-treated with or without honokiol (10 μM) or NAC (10 mM) for 1 h before treatment with H_2O_2 (500 μM) for 24 h. (A) After DAPI staining, the nuclei were observed using a fluorescence microscope (×400). (B) The percentages of apoptotic cells were determined by expressing the numbers of annexin V^+ cells as percentages. The data are expressed as mean ± SD of three independent experiments ($^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the H_2O_2-treated group).

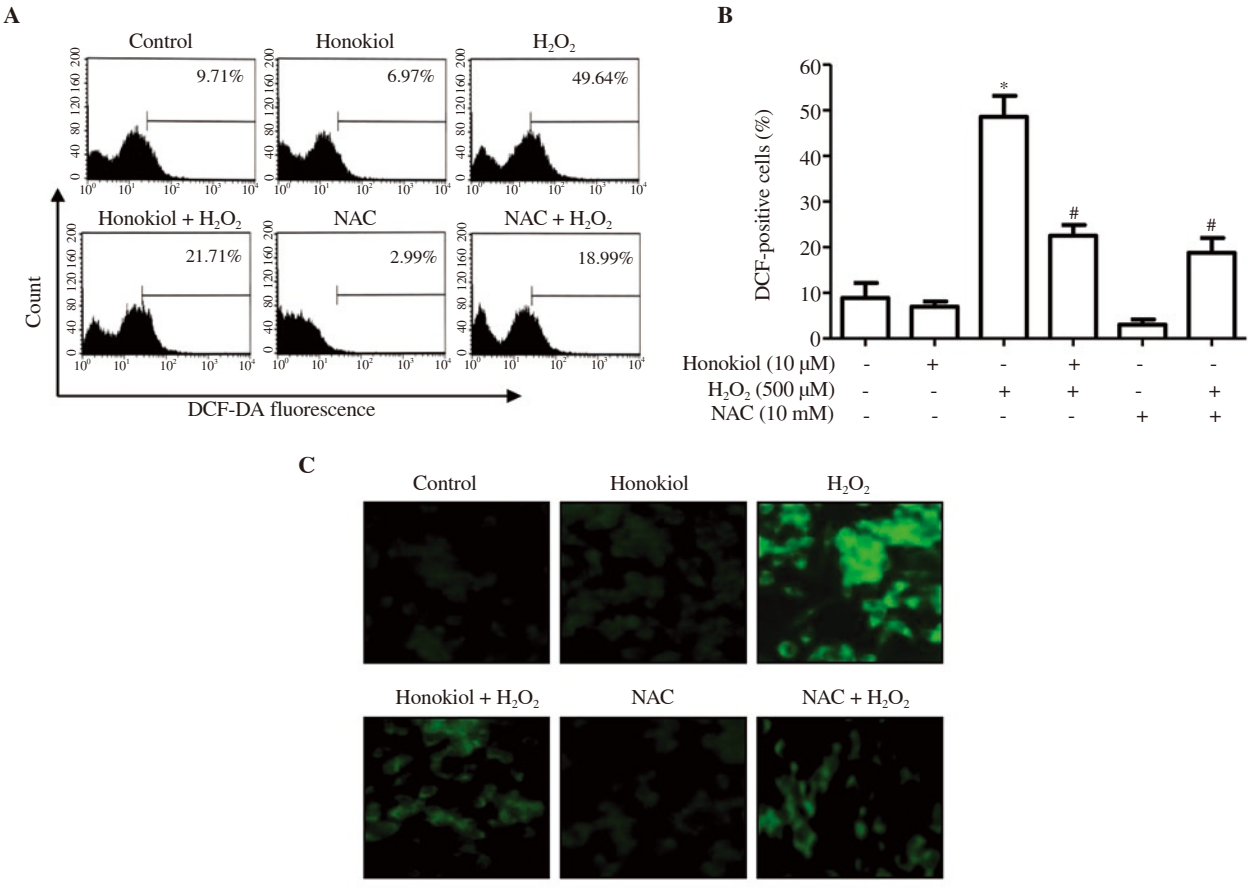

Figure 3. Inhibition of H_2O_2-induced ROS generation by honokiol in HaCaT cells. HaCaT cells were treated with 10 μM honokiol or 10 mM NAC for 1 h, and then stimulated with or without 500 μM H_2O_2 for another 1 h. (A) ROS production was measured using a flow cytometer. (B) The results are expressed as mean ± SD of triplicate determinations ($^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the H_2O_2-treated group). (C) DCF fluorescence images of cells cultured under the same conditions were captured by a fluorescence microscope (×200).

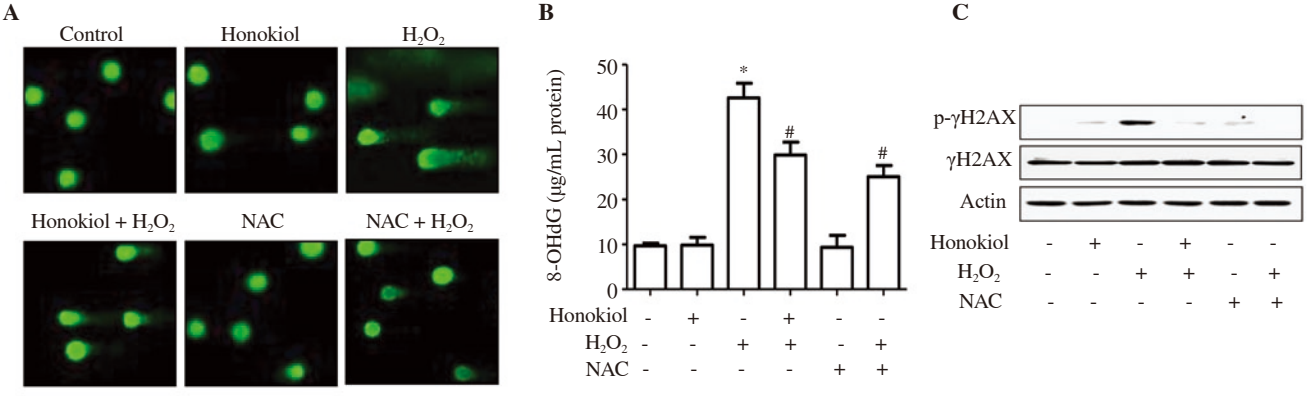

Figure 4. Attenuation of H_2O_2-induced DNA damage by honokiol in HaCaT cells. HaCaT cells were treated with or without honokiol (10 μM) or NAC (10 mM) for 1 h before treatment with H_2O_2 (500 μM) for 24 h. (A) Representative images of comet assay were presented (×400). (B) The DNA samples of cells were subjected to assessment of 8-OHdG level. The results are expressed as mean ± SD of triplicate determinations ($^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the H_2O_2-treated group). (C) p-γH2AX and γH2AX expressions were identified by Western blotting analysis.

of honokiol was further confirmed by analyzing the phosphorylation of γH2AX (p-γH2AX) and 8-OHdG production. As presented in Figure 4B and C, the level of 8-OHdG was increased more than 4 times in cells exposed to H_2O_2 compared with the control group, and the expression of p-γH2AX was also clearly increased without a significant change in total protein expression of γH2AX. However, pretreatment with honokiol or NAC could effectively attenuate H_2O_2-induced production of 8-OHdG and phosphorylation of γH2AX, indicating that honokiol can block DNA damage caused by oxidative stress.

3.5. Honokiol attenuates H_2O_2-induced mitochondrial dysfunction in HaCaT cells

To analyze whether inhibition of mitochondrial impairment is a mechanism involved in the cytoprotective effect of honokiol, MMP was estimated using JC-1 dye. As shown in Figure 5A, the ratio of JC-1 monomers in H_2O_2-treated cells was significantly increased demonstrating that H_2O_2 induced the loss of MMP. At the same time, the level of intracellular ATP content was also significantly reduced in cells treated with H_2O_2 (Figure 5B). In addition, H_2O_2 increased expression of cytochrome c in the cytoplasm, while decreasing its

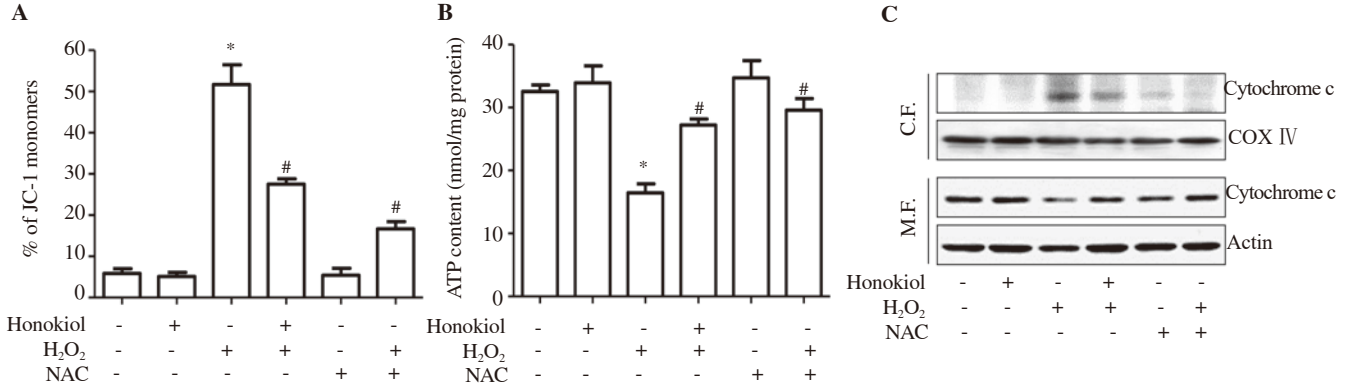

Figure 5. Protection of H_2O_2-induced mitochondrial dysfunction by honokiol in HaCaT cells. HaCaT cells were treated with honokiol (10 μM) or NAC (10 mM) for 1 h, and then exposed to H_2O_2 (500 μM) for 24 h. (A) JC-1 fluorescence intensity was detected using a flow cytometer. (B) Cellular ATP concentrations were measured. (C) Cytochrome c levels by Western blotting analysis. COX Ⅳ and actin serve as protein loading controls for the cytosol and mitochondria, respectively. All data are expressed as mean ± SD of triplicate determinations ($^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the H_2O_2-treated group). M.F.: mitochondrial fraction; C.F.: cytosolic fraction; ATP: adenosine triphoaphate.

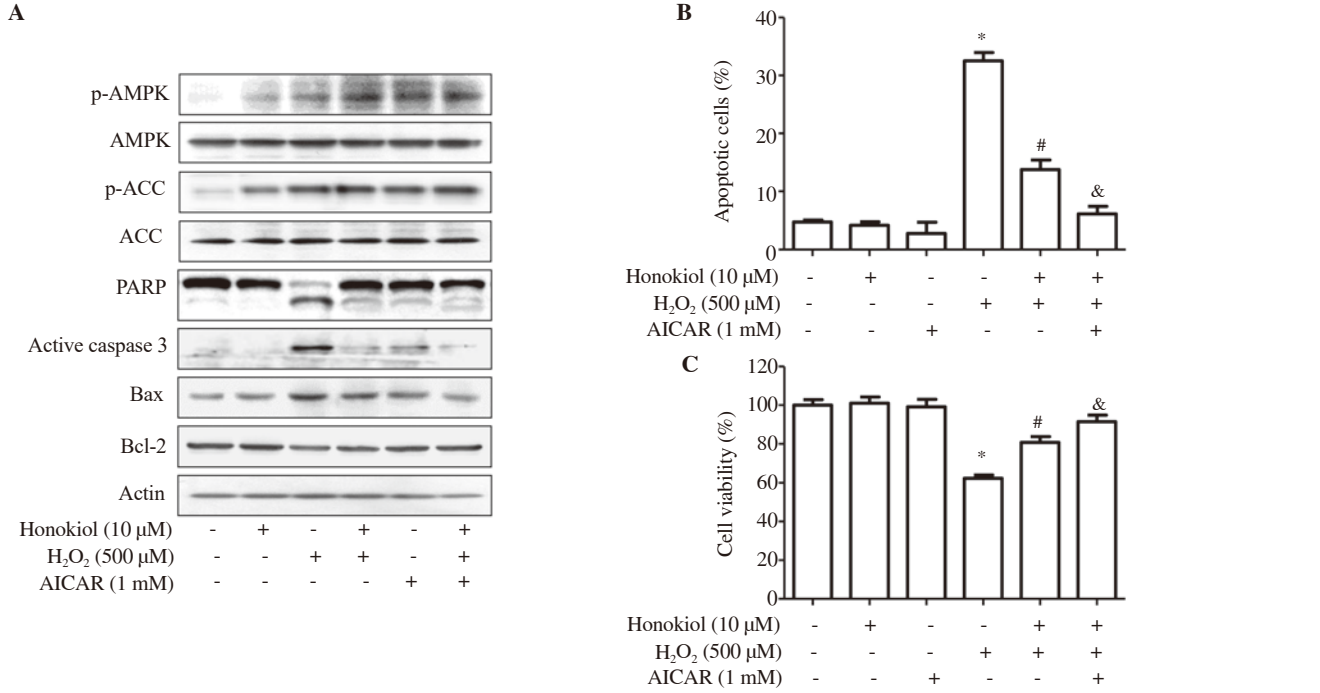

Figure 6. Activation of AMPK signaling pathway by honokiol in HaCaT cells. HaCaT cells were pre-treated with honokiol (10 μM) and/or AICAR (1 mM) for 1 h, and then treated with or without H_2O_2 (500 μM) for 24 h. (A) Equal amounts of proteins were subjected to Western blotting analysis. Actin was used as an internal control. (B) The percentages of apoptotic cells were determined by counting the percentages of annexin V^+ cells. (C) The cell viability was determined by MTT assay. All data are presented as mean ± SD obtained from three independent experiments ($^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the H_2O_2-treated group; $^{\&}P < 0.05$ compared with the honokiol and H_2O_2-treated group). AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide.

expression in the mitochondria (Figure 5C). However, pretreatment with honokiol reversed these changes.

3.6. Honokiol promotes activation of the AMPK signaling pathway in HaCaT cells

Immunoblotting results showed that expression of phosphorylated AMPK (p-AMPK) in cells treated with honokiol or H_2O_2 alone was slightly increased compared with the untreated control without significant changes in its total protein expression (Figure 6A). Interestingly, this induction was largely increased by co-treatment with honokiol and H_2O_2 or in the presence of an AMPK activator, AICAR. At the same time, p-ACC, a downstream target protein of p-AMPK, was similarly upregulated. In addition, anti-apoptotic Bcl-2 was downregulated while pro-apoptotic Bax was upregulated in cells treated with H_2O_2, and activation of caspase-3 and degradation of PARP were significantly increased by H_2O_2 treatment (Figure 6A). However, these changes were markedly abolished in cells pre-treated with honokiol, and these protective effects of honokiol were enhanced in the presence of AICAR. Concomitant with these results, honokiol effectively reversed H_2O_2-induced apoptosis and showed anti-proliferative effects. AICAR further improved the cytotoxic inhibitory effect of honokiol (Figures 6B and C).

4. Discussion

Mitochondria are most vulnerable to excessive H_2O_2 insults among intracellular organelles, and their dysfunction contributes significantly to ROS production, causing oxidative damage to cellular components. In addition, excessive ROS levels can induce modifications of nucleic acids such as purines and pyrimidines and destroy DNA strands, causing DNA damage, and ultimately contributing to cell death[31,32]. In the present study, H_2O_2 was utilized to induce oxidative damage and it was found that H_2O_2 lowered HaCaT cell viability by triggering DNA damage and mitochondria-mediated apoptosis through promoting ROS production. However, honokiol was found to have the ability to alleviate H_2O_2-mediated cytotoxicity, DNA damage, and apoptotic cell death, while having ROS scavenging activity.

Apoptosis is a physiological form of cell death that allows tissue remodeling and homeostasis and removes unnecessary cells from organisms. Cells undergo apoptosis through two main pathways, distinguished by extrinsic and intrinsic pathway[33,34]. The former can be activated when death receptors are bound to corresponding ligands present on the cell membrane surface, which ultimately activate caspase-8. On the other hand, the latter is accompanied by mitochondrial dysfunction, which results in the cytosolic release of apoptogenic factors, especially cytochrome c, and the formation of apoptotic complexes with the cytoplasmic proteins Apaf-1 and ATP, leading to caspase-9 activation. Both pathways converge with the activation of effector caspases such as caspase-3 and caspase-7 to break down various cellular structures through cleavage of specific substrates, including PARP, eventually completing apoptosis[35,36]. The overload ROS disrupts the mitochondrial phospholipid bilayer, which causes depolarization of the mitochondrial membrane, ultimately resulting in the loss of MMP[33,34]. During this process,

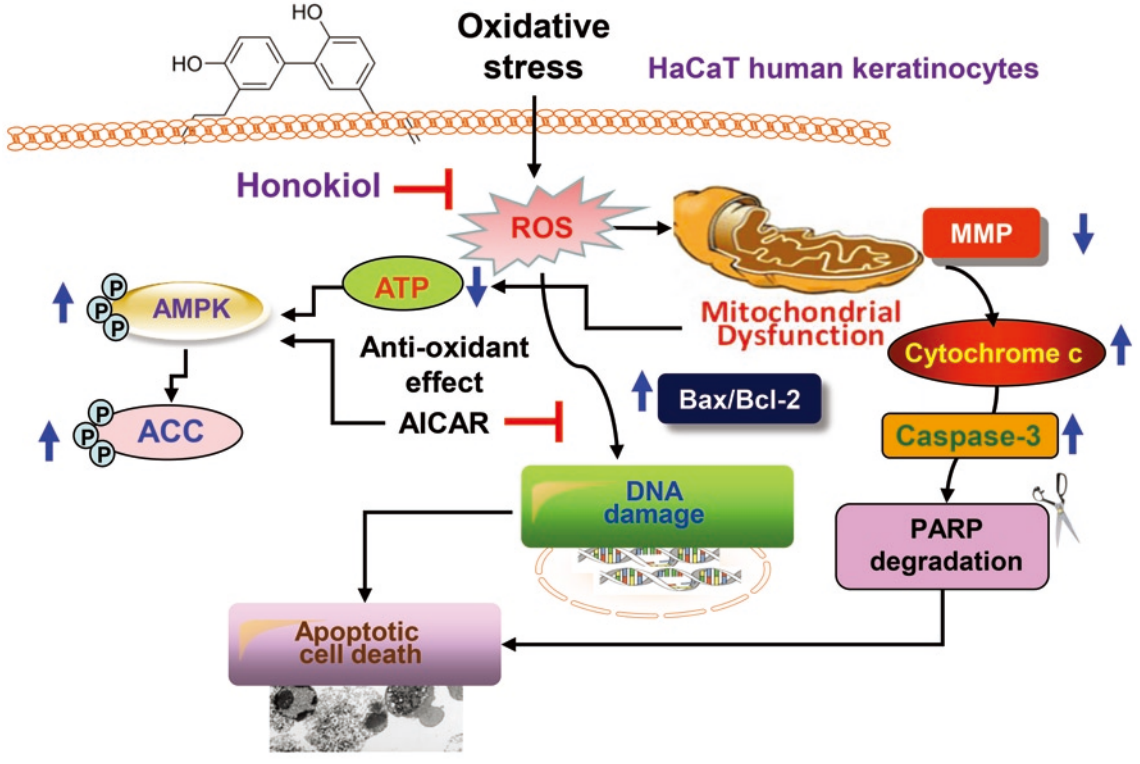

Figure 7. Protective effects of honokiol against oxidative stress-induced cytotoxicity are associated with the activation of AMPK signaling pathway in HaCaT cells.

the permeability of the mitochondrial membranes increases, allowing apoptogenic factors in mitochondrial intermembrane space to be released into the cytosol. In addition, mitochondrial dysfunction accompanies the shrinkage of the cristae membranes, where the protein complexes of the electron transport chain are located, ultimately reducing ATP production[33,37]. To evaluate the preventive effect of honokiol on H_2O_2-induced mitochondrial dysfunction, I examined MMP, ATP content, and cytochrome c expression. The results indicated that H_2O_2 caused changes on ATP, MMP and cytosolic cytochrome c expressions. However, honokiol significantly diminished the H_2O_2-induced disruption of MMP and maintained cytochrome c in mitochondria during H_2O_2 exposure, followed by significant ATP restoration.

The activation of caspase cascade is tightly regulated by the expression of diverse regulators. Among them, Bcl-2 family proteins are divided into two subgroups, pro- and anti-apoptotic proteins, according to their structural and functional homology[33-36]. As a representative pro-apoptotic protein, Bax, which is present in the outer membrane of the mitochondria, induces the release of apoptogenic factors from the mitochondria to the cytoplasm by promoting mitochondrial permeability transition or weakening the barrier function of the mitochondria outer membrane. Conversely, anti-apoptotic proteins, such as Bcl-2, are essential for maintaining mitochondrial permeability and membrane barrier stabilization. Bcl-2 also functions as a reaction force to suppress cellular damage by diminishing lipid peroxidation of the mitochondrial membrane caused by ROS[35,37]. In this study, the Bax/Bcl-2 expression ratio was enhanced, and the activation of caspase-3 and degradation of PARP were also obviously increased in H_2O_2-treated HaCaT cells, which are consistent with previous studies[38,39]. However, these changes were all markedly reversed by honokiol, indicating that honokiol can protect HaCaT cell from apoptosis by inhibiting the intrinsic apoptosis pathway activated by oxidative stress.

Accumulating evidence indicates that activation of AMPK serves as a pivotal cellular energy sensor and a master energy regulator in cells. AMPK is activated through phosphorylation at threonine 172 to protect cells from various metabolic stresses in low energy conditions, especially when the ratio of AMP/ATP or ADP/ATP is increased[19,20]. Once activated, AMPK blocks the biosynthetic pathway to increase the preservation of ATP and the expression or activity of proteins involved in catabolism, including ACC. It has also been found that various natural compounds act as activators of AMPK to prevent oxidative damage in keratinocytes[17,40,41]. Moreover, artificially blocking AMPK expression and activity increased mitochondrial dysfunction, ATP levels, and apoptosis due to oxidative stress, but AMPK overexpression had the opposite effect[23,24,40-42]. Therefore, whether AMPK acts as a potential signaling molecule in blocking oxidative stress by honokiol was evaluated in this study. According to the results of immunoblotting, increased phosphorylation of AMPK and ACC was observed in response to treatment with honokiol, H_2O_2, and AICAR compared with control cells, respectively, and their phosphorylation was further increased in cells treated with honokiol and H_2O_2. In addition, the increased apoptosis and decreased cell viability caused by H_2O_2 treatment were significantly reversed by pretreatment with honokiol. Further, the cytotoxic protective effect of honokiol against H_2O_2 is enhanced in the presence of AICAR. These results implicated that under oxidative conditions, the protective effect of honokiol on H_2O_2-stimulated HaCaT cells could be attributed to activation of the AMPK signaling pathway. These data support well with previous studies that activation of the AMPK signaling pathway is involved in the potential antioxidant effect of honokiol[21-25].

In summary, the current study elucidated the protective effect of honokiol against H_2O_2-induced oxidative damage and explored the mechanism of action in HaCaT human keratinocytes. According to our findings, honokiol reversed the increased intracellular ROS generation and mitochondrial damage induced by H_2O_2, eventually inhibiting DNA damage and apoptosis. In this process, honokiol activated AMPK, which was associated with the restoration of reduced ATP induced by H_2O_2. The current data suggest that honokiol prevents oxidative stress-induced keratinocyte damage *via* the activation of AMPK signaling pathway (Figure 7). However, further studies are needed to evaluate how honokiol can modulate the activation of AMPK and whether other upstream signaling pathways can intervene in the antioxidant activity of honokiol.

Conflict of interest statement

I declare that there is no conflict of interest.

Author's contributions

YHC designed experiments, conducted the experiments, interpreted the results, wrote and finalized the manuscript.

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