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Metformin Prevents H_2O_2-Induced Senescence in Human Lens Epithelial B3 Cells

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Background: The primary purpose of this study was to investigate the protective effect of metformin against hydrogen peroxide (H_2O_2)-induced cellular senescence and to explore the underlying molecular mechanism of lens epithelial cell senescence.

Material/Methods: We used H_2O_2 to establish senescence in human lens epithelial B3 cells. The cells were exposed to H_2O_2 for different numbers of days to mimic aging. Senescence was assessed by senescence-associated β-galactosidase staining, and the molecular mechanism was assessed by real-time polymerase chain reaction (RT-PCR) and western blot analysis. The cultured cells were exposed to 150 μM H_2O_2 for 7 days with or without metformin to detect the underlying molecular mechanism of lens epithelial cell senescence.

Results: The lens epithelial cells exposed to 150 μM H_2O_2 for 7 days exhibited senescence. The expression levels of senescence-related markers were increased in H_2O_2-treated cells. Metformin prevented H_2O_2-induced cellular senescence in human lens epithelial B3 cells.

Conclusions: These findings suggest that senescence marker expression is increased in the cells exposed to H_2O_2. Metformin protects human lens epithelial B3 cells from H_2O_2-induced senescence.

MeSH Keywords: Cataract • Cell Aging • Epithelial Cells • Metformin

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Background

Cataract is the current leading global cause of blindness among the elderly population, and age is by far the greatest risk factor for cataract [1,2]. Age-related cataract (ARC) formation is characterized by a gradual increase in lens opacity with increasing age. In addition to aging, other risk factors, including ultraviolet radiation, diabetes, smoking and genetic factors, have been identified by epidemiological studies [3–6]. Oxidative stress is thought to be a major mechanism by which ARCs are formed and develop [7,8]. Under some circumstances, the level of stress determines how a cell responds to damage. Apoptosis is a response to overwhelming stress, whereas senescence is a consequence of less severe damage [9]. Persistent oxidative stress leads to the functional degradation of cells, of which cellular senescence is an important hallmark [10,11]. Apoptosis of lens epithelial cells (LECs) has been recognized as the cellular basis of cataract formation [12]. However, the association between the appearance of cataract and LEC senescence has been only poorly described.

Aging, a universal biological phenomenon, is defined as a time-dependent decline in physiological functional [13,14] associated with the development of age-related diseases. Cellular senescence, the hallmark of aging [15], is thought to play an important role in age-related disease. Senescent cells are characterized by several features, including elevated senescence-associated β-galactosidase (SA-β-gal) activity and the senescence-associated secretory phenotype (SASP) [16]. Two types of cellular senescence have been described: telomere-dependent replicative senescence and stress-induced premature senescence (SIPS) [17]. Several *in vitro* treatments that promote cellular senescence and induce oxidative SIPS have been identified. For example, the oxidative stressor hydrogen peroxide (H_2O_2) can produce an oxidative environment that rapidly leads to senescence [18] and can be used to establish a senescence model and probe the aging mechanism. When cellular senescence is induced under various conditions, senescent cells display certain characteristics. Some biomarkers reflect activation of the senescence mechanism [17].

Metformin (Met), a first-line drug used to treat diabetes mellitus, has recently been shown to protect against cancer [19,20], cardiovascular disease and aging-related diseases [21,22] and has become the first anti-aging drug in clinical trials. Smieszek et al. confirmed that Met reduced the expression of oxidative stress markers in mOECs [23]. Senolytic and antioxidative properties of Met were also shown in some studies, which is crucial for oxidative homeostasis [23–25]. Met was suggested to extend the lifespan of multiple species [25–28], simultaneously improving the general fitness of the subjects. A study on aging found that Met treatment delayed the onset of ARC formation [25]. To date, few studies have shown the preventative effects of Met against age-related eye diseases. The association between ARC formation and aging markers has been reported [29,30], but the specific mechanism by which cellular senescence causes cataract remains largely unknown. In the present study, we explored whether Met treatment could attenuate human lens epithelial B3 cells (HLE-B3) senescence due to H_2O_2 exposure.

Material and Methods

Cell treatment

HLE-B3 cells (American Type Culture Collection, Manassas, VA, USA) were obtained from the Department of Ophthalmology, Eye and ENT Hospital of Fudan University. The HLE-B3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Hyclone, GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS; Gibco, South America), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, USA) in a humidified 5% CO_2 atmosphere at 37°C. The medium was changed every 3 days. Cells were detached from culture flask using trypsin (Gibco, USA), counted, seeded in 6-well plates and incubated overnight. Then, the cells were treated with 0, 50, 75, 100, 150, or 200 μM H_2O_2 for different numbers of days. To study senescence, the cells treated with 150 μM H_2O_2 were incubated with Met (Sigma-Aldrich) at different concentrations (0.5, 1.0, 2.0 mM) for 7 days. The incubation without Met was used as control.

SA-β-gal staining

SA-β-gal activity was evaluated by using a Senescence-Associated β-Galactosidase Staining kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. HLE-B3 cells seeded in 6-well plates were washed with phosphate-buffered saline (PBS), fixed for 15 minutes and then washed 3 times. The cells were incubated with β-galactosidase staining solution at 37°C overnight. The cells were washed twice with PBS and then observed and photographed with an inverted microscope (Nikon ECLIPSE Ti). The cells of each group were counted by using ImageJ software. The percentage of positive cells in total cells was assessed by counting 1000 cells in 7 random fields, for each group. The experiment was performed 3 times.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted from HLE-B3 cells using TRIzol reagent (Ambion, USA). RNA was reverse transcribed into cDNA using reverse transcriptase, reverse transcriptase buffer, and oligo(dT)15 primers from Promega Corporation (USA) and dNTPs and $MgCl_2$ from Takara Bio, Inc. The obtained cDNA was used for real-time quantitative polymerase chain reaction (q-RT-PCR) by

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Table 1. Primer sequences in our experiment.

Gene	Primer	Length of production	Gene ID
p21	F 5'-CGATGGAACTTCGACTTTGTCA-3'	219 bp	NM_000389.5
	R 5'-GCACAAGGGTACAAGACAGTG-3'		
p16	F 5'-GAGCAGCATGGAGCCTTC-3'	126 bp	NM_000077.4
	R 5'-GGCCTCCGACCGTAACTATT-3'		
IL-6	F 5'-ACTCACCTCTTCAGAACGAATTG-3'	149 bp	NM_000600.5
	R 5'-CCATCTTTGGAAGGTTCAGGTTG-3'		
IL-8	F 5'-TTGGCAGCCTTCCTGATTTC-3'	248 bp	NM_000584.4
	R 5'-AACTTCTCCACAACCCTCTGCA-3'		
FAS	F 5'-GTGAGGGAAGCGGTTTACGA-3'	193 bp	NM_000043.6
	R 5'-AGATGCCCAGCATGGTTGTT-3'		

using an Applied Biosystems StepOne Real-Time PCR system in accordance with the manufacturer's protocol and SYBR Green Master Mix (TOYOBO Co., Ltd.). The volume of each reaction was 20 μL. The sequences of the specific primers used in our experiment are shown in Table 1. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. RT-PCR amplification of each primer was performed in triplicate to verify the results [31].

Western blotting

Treated cells were harvested and lysed in lysis buffer (Beyotime Institute of Biotechnology) containing 1% protease inhibitors for 30 minutes at 4°C. The lysate was centrifuged, and the supernatant was obtained. The protein concentration was calculated using an Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). The cellular proteins were separated by electrophoresis on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies diluted in PBS containing 5% bovine serum albumin (BSA) and 0.1% Tween 20 (AMRESCO, USA) at 4°C overnight. The primary antibodies used were anti-p21 (1: 1,500 dilution, ab109520, Abcam), anti-p53 (1: 500 dilution, 2524), anti-pAMPKα (Thr172, 1: 750 dilution, 2535), anti-AMPKα (1: 500 dilution, 2532), anti-pACC (Ser79, 1: 750 dilution, 3661), and anti-acetyl-CoA carboxylase (ACC; 1: 750 dilution, 3662), all of which were from Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-β-actin (Sigma). After being washed with Tris-HCl buffered solution containing 0.1% Tween 20 (TBST), the membranes were incubated with an appropriate secondary antibody (1: 2500 dilution, ab6721 or ab6728, Abcam) for 1 hour at 37°C. The bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (ECL) (Millipore Corporation, Billerica, MA, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad Software). All data were exported to GraphPad Software for statistical analyses. Statistical significance was determined based on *P*-values obtained by Student's *t*-test.

Results

Establishment of an HLE-B3 cell senescence model

H_2O_2 is the most commonly used inducer of LEC senescence and cataract [32,33]. Several studies have shown that H_2O_2 can cause damage to LECs, resulting in subsequent cataract development [34,35]. In the present study, we exposed cultured HLE-B3 cells to different low doses of H_2O_2 for 3 to 7 days to stimulate oxidative stress and induce senescence. The optical concentration and treatment time were determined by SA-β-gal staining. By 5 to 7 days after exposure to H_2O_2, HLE-B3 cells exhibited the morphological characteristics of senescent cells and had become large and flattened (Figure 1A). Positive staining was observed, and positively stained cells were counted (Figure 1B). The percentage of SA-β-gal-positive cells was significantly increased in the groups treated with H_2O_2 for 7 days compared to the control group (Figure 1C, 1D). We used HLE-B3 cells treated with 150 μM H_2O_2 for 7 days as a senescence model in subsequent studies.

The cell cycle regulatory proteins p53 and p21 have been reported to play roles in cell senescence [36,37]. To assess the cellular and molecular basis of senescence in HLE-B3 cells, we detected the expression levels of the senescence-related

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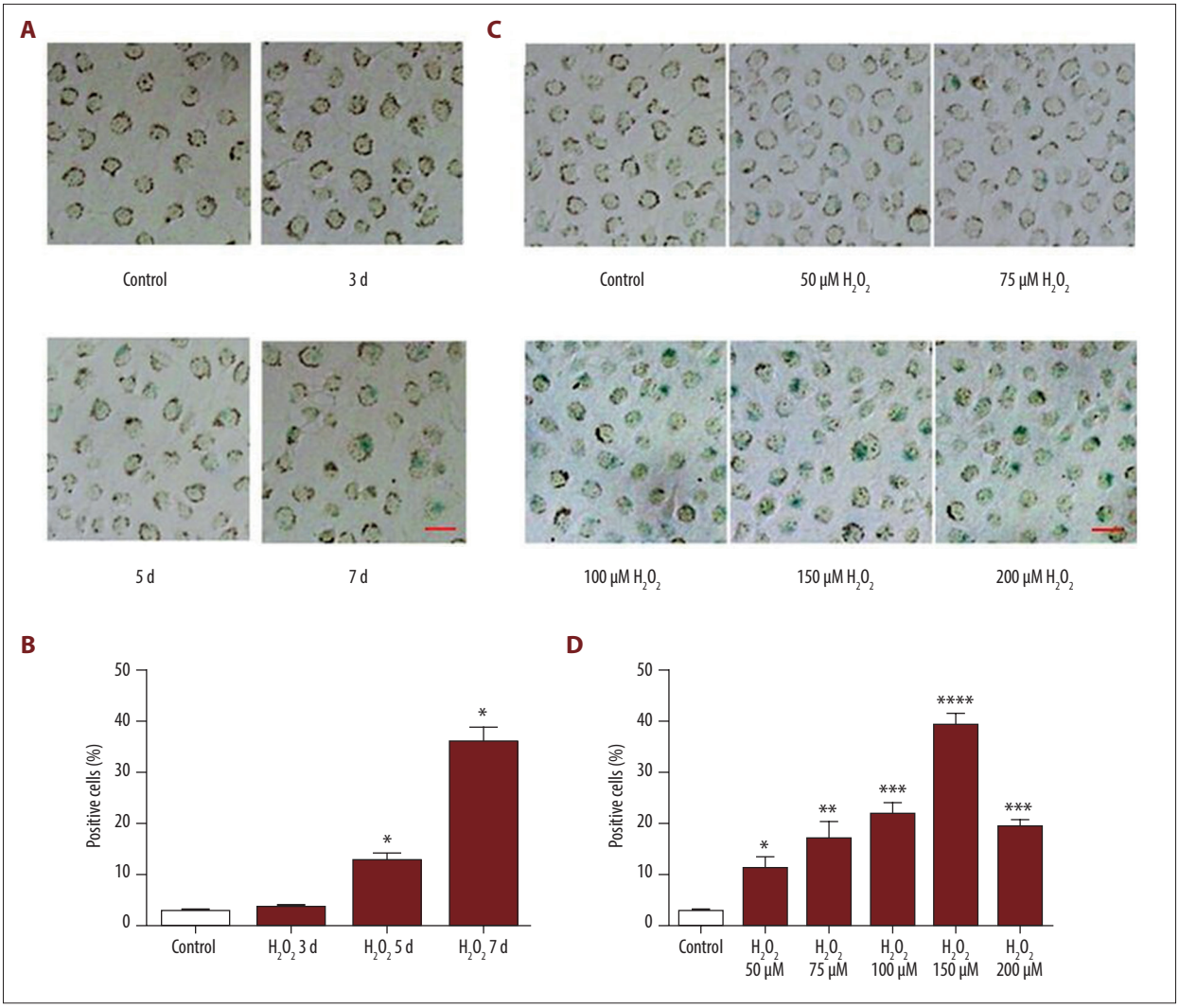

Figure 1. Hydrogen peroxide (H_2O_2) induces senescence in HLE-B3 cells. (**A**) The images show senescence-associated β-galactosidase (SA-β-gal)-positive HLE-B3 cells treated with 150 μM H_2O_2 for 3, 5, and 7 days. (**B**) The histogram shows the percentage of SA-β-gal-positive HLE-B3 cells treated with H_2O_2 for different numbers of days. (**C**) The images show SA-β-gal-positive HLE-B3 cells treated with different concentrations of H_2O_2 for 7 days. (**D**) The histogram shows the percentage of SA-β-gal-positive HLE B-3 cells treated with different concentrations of H_2O_2 for 7 days. Scale bar: 20 μm.

biomarkers p21 and p53 and found that they were significantly increased in cells treated with H_2O_2 (Figure 2A, 2B). To verify the establishment of senescence in our H_2O_2-induced senescence model, we evaluated the expression of a series of senescence markers. The expression levels of the most common cell aging biomarkers, p53, p21, and p16 [36], were all increased in H_2O_2-treated cells (Figure 2B, 2C). The proinflammatory cytokines interleukin (IL)-6 and IL-8 are thought to be associated with senescence [38]. Therefore, we quantified IL-6 and IL-8 and found that their levels were also increased in H_2O_2-induced senescent cells (Figure 2D). These results indicated that the oxidative stress-induced cellular aging model had been successfully established. Data are presented as the mean±standard error of the mean (SEM) (n=3 per group); * P<0.05 significantly different from the control, ** P<0.01, *** P<0.001. The bar represents 20 μm.

AMPK was inactivated in H_2O_2-induced senescent HLE-B3 cells

As AMPK activation has been reported to decline with age [39], we tested this finding in our senescence model. The levels of pAMPKα (Thr172) and its downstream substrate, ACC (pACC; at Ser79), were significantly decreased in HLE-B3 cells treated with H_2O_2, whereas the protein levels of AMPKα and ACC remained unchanged (Figure 3A, 3B). AMPK is believed to play a central role in controlling lipid metabolism, and AMPK activation can result in the inhibition of fatty acid synthase (FAS) [40].

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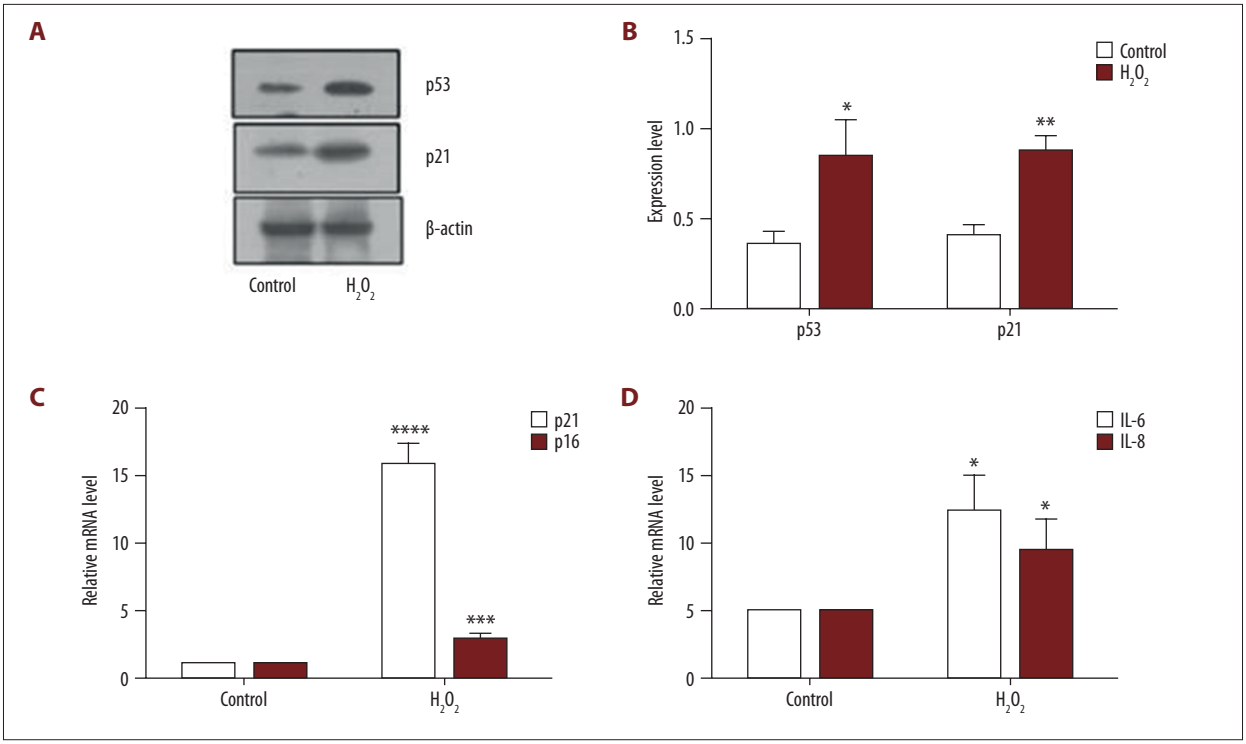

Figure 2. Senescence-related marker expression in HLE-B3 cells (**A**) The protein levels of p21 and p53 in HLE-B3 cells exposed to control or hydrogen peroxide (H_2O_2) treatment for 7 days. (**B**) Statistical analysis of the protein levels of p53 and p21 in the control and H_2O_2-treated groups. (**C**) The mRNA expression of the senescence markers p21 and p16 in HLE-B3 cells was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). (**D**) Gene expression levels of the inflammatory cytokines IL-6 and IL-8 were determined by qRT-PCR. The values are normalized to those of β-actin and are shown relative to those of control cells. Data are presented as the mean±standard error of the mean (n=3 per group).

In the present study, we detected AMPK inactivation and found the expression level of the AMPK target gene FAS to be increased in senescent cells (Figure 3C). Taken together, these data indicate that the AMPK pathway is inhibited in H_2O_2-induced senescent cells.

Met inhibited H_2O_2-induced senescence in HLE-B3 cells

Studies have shown that Met exerts multiple antiaging effects at the cellular and organismal levels [26]. In the present study, we observed the effect of Met on H_2O_2-induced senescence and explored the molecular mechanism of LEC senescence. HLE-B3 cells were cultured in H_2O_2 alone or with 0.5 mM, 1.0 mM, or 2.0 mM Met for 7 days, followed by SA-β-gal staining. The percentage of SA-β-gal-positive HLE-B3 cells was significantly decreased in the groups treated with Met (Figure 4A, 4B). We chose 1.0 mM as the optimal concentration of Met for subsequent research. To further examine whether Met, an AMPK activator, inhibits H_2O_2-induced senescence in HLE-B3 cells, we examined the expression of age-related markers that were altered by exposure to H_2O_2 in HLE-B3 cells treated with Met. We found that the expression of p53 and p21 was significantly reduced in the Met group (Figure 4C). Moreover, we detected that the mRNA expression of the aging-related genes p21 and p16 was decreased (Figure 4D), and the mRNA expression levels of the proinflammatory cytokines IL-6 and IL-8 were also markedly reduced in the Met group compared with the control group (Figure 4E).

Met inhibited H_2O_2-induced senescence via AMPK activation

To examine whether AMPK was involved in the Met-mediated inhibition of H_2O_2-induced senescence, we next examined the protein levels of total AMPKα, pAMPKα (Thr172) and its downstream target, pACC (Ser79). Met treatment significantly increased the relative protein levels of pAMPKα (Thr172), which led to increased phosphorylation of its direct target, ACC, at Ser79, as shown in Figure 5A and 5B. In addition, expression of the AMPK target gene FAS was decreased in cells cultured with Met (Figure 5C).

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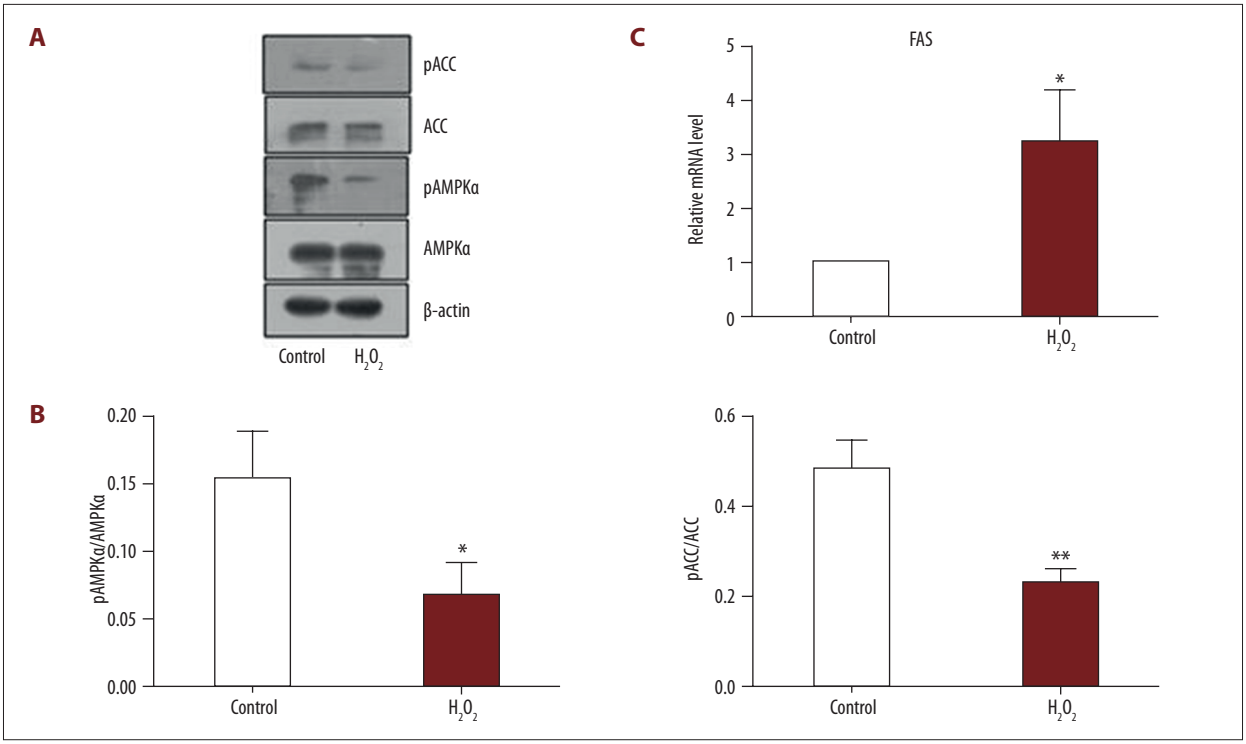

Figure 3. The AMPK activity is inhibited in hydrogen peroxide (H_2O_2)-treated HLE-B3 cells. Cells in the control group were incubated in complete medium, and cells in the senescence group were cultured in medium with H_2O_2 for 7 days. (**A**) Western blots showing the protein levels of pAMPKα (Thr172), AMPKα, pACC (Ser79), ACC, and β-actin. (**B**) Histogram summarizing the outcome of statistical analysis of the western-blot gray values. (**C**) The relative expression of the AMPK target gene FAS at the mRNA level was detected by quantitative real-time polymerase chain reaction; the values were normalized to β-actin and are shown relative to those in control cells. The data are presented as the mean±standard error of the mean (* P<0.05, ** P<0.01; n=3).

Discussion

LECs are a single layer of epithelial cells located anterior to the capsular membrane of the lens. The normal construction and function of LECs are crucial to maintain the transparency of the entire lens. When the function of the LECs changes, the transparency of the lens may decrease, resulting in subsequent cataract development [41]. In the present study, we used HLE-B3 cells to induce senescence *in vitro* to study the underlying molecular mechanism of LEC senescence. Oxidative stress caused by reactive oxygen species (ROS) has been recognized as an important mediator of ARC pathogenesis [41–43]. H_2O_2 is a major intracellular ROS that accumulates in substantial amounts in the lens, eventually causing damage to LECs, leading to the subsequent initiation and progression of cataract [43]. Therefore, we used H_2O_2 to construct a senescence model to mimic the characteristics of cataract pathogenesis *in vivo* to explore the protective effects of Met and its probable mechanism. Our study is the first to demonstrate the protective effect of Met against H_2O_2-induced senescence in HLE-B3 cells.

By SA-β-gal staining, we found that H_2O_2-treated HLE-B3 cells showed high SA-β-gal activity, indicating aging cells. Cellular senescence is defined as stable arrest of the cell cycle involving several cellular changes [17]. Senescent cells exhibit a number of specific characteristics and express senescence markers. The cyclin-dependent kinase (CDK) inhibitor p21 is a major transcriptional target of p53; p21 transcription is activated by p53, which inhibits CDK, resulting in cell cycle arrest. Our results revealed that the expression of p53 and p21 was markedly increased in H_2O_2-induced HLE-B3 senescent cells. In addition, p16-mediated senescence acts through the retinoblastoma (Rb) pathway, suppressing CDK and leading to cell cycle arrest. The cell cycle inhibitors p53, p21, and p16 have been used as reliable senescence markers reflecting activation of the senescence program [11]. A higher p16 expression level was observed in senescent HLE-B3 cells than in control cells. Another important feature of senescence is the formation of a so-called SASP, which is characterized by the increased expression and secretion of proinflammatory cytokines, chemokines, growth factors, and proteases [11]. The most prominent cytokines of the SASP are IL-6 and IL-8. In this study, we confirmed that the expression levels of these cytokines, which are

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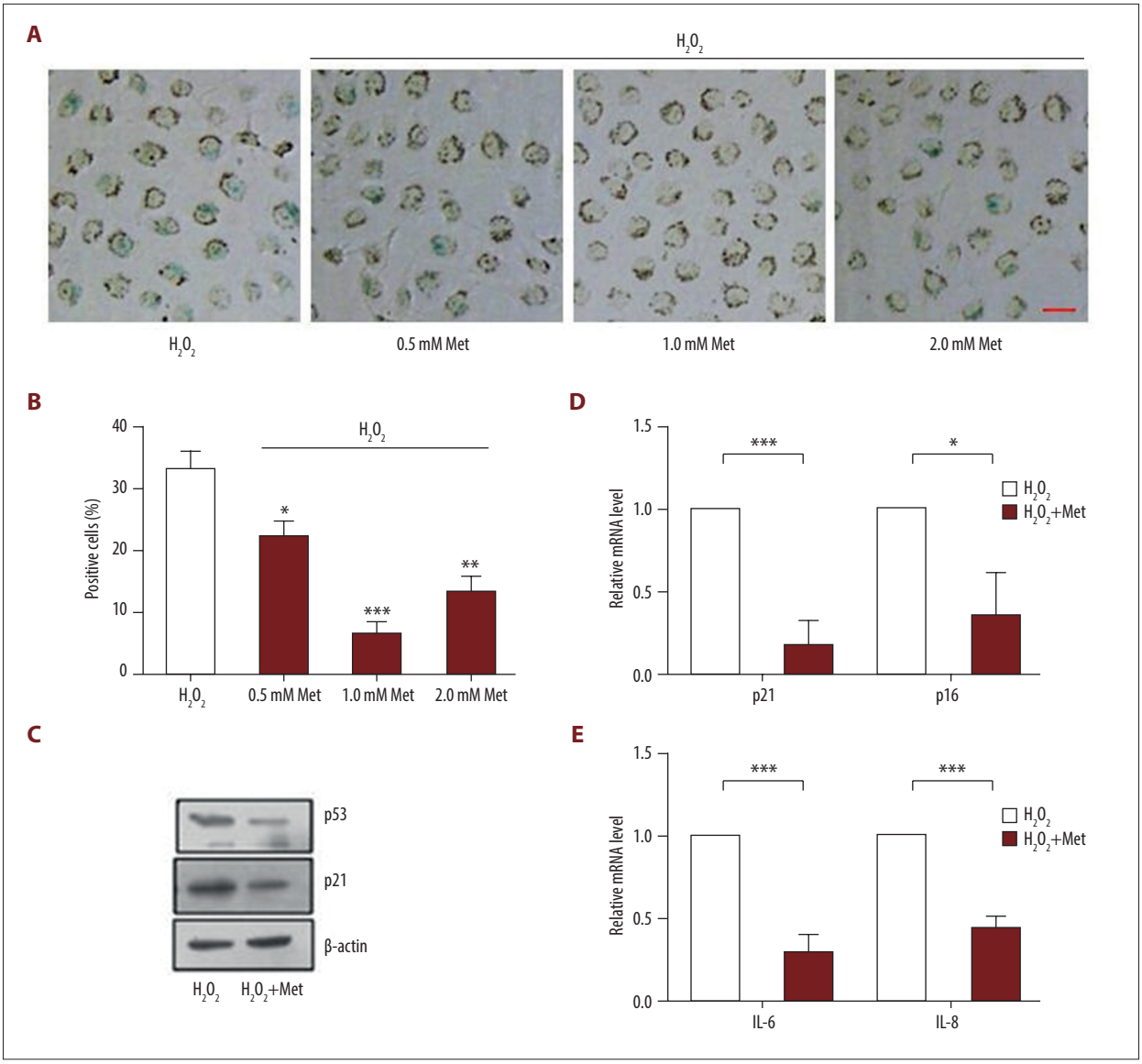

Figure 4. Metformin (Met) reduces senescence induced by hydrogen peroxide (H_2O_2) in HLE-B3 cells. (**A**) Representative images showing senescence-associated β-galactosidase (SA-β-gal)-positive HLE-B3 cells treated with different concentrations of Met for 7 days. The bar represents 20 μm. (**B**) Quantitative analysis of SA-β-gal-positive cells. (**C**) The protein levels of p53 and p21 in the Met group compared with the control group. (**D**) The mRNA expression levels of p21 and p16 after treatment with 1.0 mM Met were assessed by quantitative real-time polymerase chain reaction (qRT-PCR). (**E**) The mRNA expression levels of interleukin (IL)-6 and IL-8 after treatment with 1.0 mM Met were determined by qRT-PCR. The values were normalized to those of β-actin and are shown relative to those in H_2O_2-treated cells. Data are presented as the mean±standard error of the meant (* P<0.05, ** P<0.01, *** P<0.001; n=3).

the most widely used senescence markers, were increased in H_2O_2-treated HLE-B3 cells. This finding may contribute to exploring the molecular mechanisms of LEC senescence.

Met, an extensively studied antiaging agent, has demonstrated protective effects against several age-related diseases [22,44]. Met can delay aging via inhibition of the inflammatory pathway and activation of AMPK to regulate oxidative stress [25,45].

In this study, we used Met to investigate the molecular basis of LEC senescence. We found that Met-treated HLE-B3 cells showed lower SA-β-gal activity. After senescent HLE-B3 cells were treated with Met, p53, p21, p16, IL-6, and IL-8 were significantly downregulated. We confirmed that Met protected H_2O_2-treated HLE-B3 cells against senescence.

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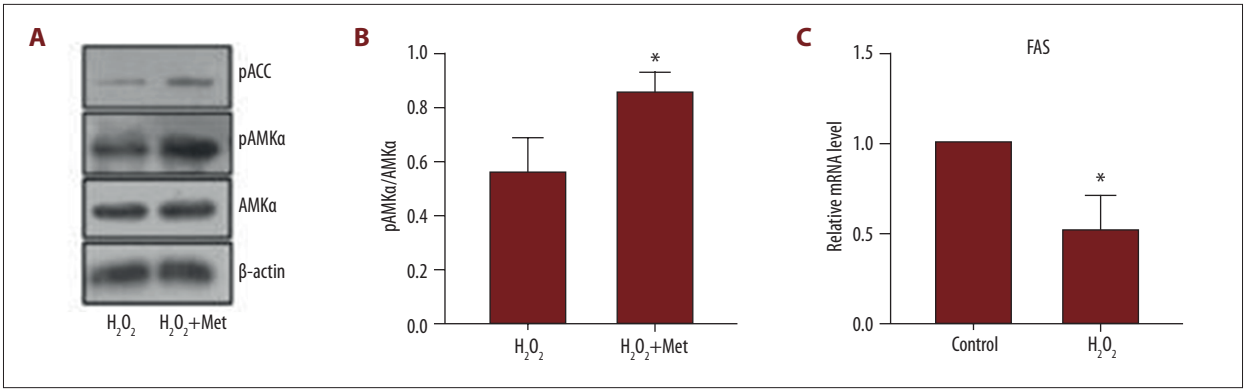

Figure 5. The AMPK pathway mediates the protective effects of metformin (Met) in hydrogen peroxide (H_2O_2)-treated HLE-B3 cells. (**A**) The protein levels of pAMPKα (Thr172), AMPKα, and pACC (Ser79) were examined after treatment with 1.0 mM Met. (**B**) The ratio of pAMPKα to total AMPKα was analyzed using a histogram. (**C**) The mRNA expression of FAS was detected by real-time polymerase chain reaction. Data are presented as the mean±standard error of the mean (* P<0.05, n=3).

Met is frequently used as an AMPK agonist in biochemical studies. AMPK, which is composed of a catalytic α subunit and regulatory β and γ subunits, serves as a cellular energy sensor [46]. Phosphorylation of the catalytic α subunit at Thr172 is required to activate AMPK. AMPK lies upstream of multiple signaling pathways known to modulate the aging process [47]. Met is frequently used as an AMPK agonist in biochemical studies. AMPK, which is composed of a catalytic α subunit and regulatory β and γ subunits, serves as a cellular energy sensor [46]. Phosphorylation of the catalytic α subunit at Thr172 is required to activate AMPK. AMPK lies upstream of multiple signaling pathways known to modulate the aging process [47]. This finding indicates that AMPK inactivation may be involved in LEC senescence and is associated with the formation of ARC. Met prevents H_2O_2-induced senescence in an AMPK activation-dependent manner. Several studies have shown the role of antioxidant protection by Met [23–25], but the underlying molecular mechanism and the association with the effect on LECs remains to be further explored. The present study is the first to investigate the effect of Met on H_2O_2-induced HLE-B3 cell senescence and the first to report that AMPK inactivation may be responsible for LEC senescence.

Conclusions

Our data revealed that Met prevents H_2O_2-induced senescence in HLE-B3 cells. We speculated that Met can be exploited as a potentially useful drug for cataract prevention. We also found that the expression of a series of age-related markers (the CDK inhibitors p53, p21, and p16 and the inflammatory cytokines IL-6 and IL-8) was increased in the H_2O_2-induced senescence model; these markers may be therapeutic targets for the prevention of ARC formation. The limitation of the present study is that the exact protective mechanism of Met on LEC senescence need to elucidate further *in vivo*.

Conflicts of interest.

None.

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