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Sulforaphane Attenuates H_2O_2-induced Oxidant Stress in Human Trabecular Meshwork Cells (HTMCs) via the Phosphatidylinositol 3-Kinase (PI3K)/Serine/Threonine Kinase (Akt)-Mediated Factor-E2-Related Factor 2 (Nrf2) Signaling Activation

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: The aim of this study was to investigate whether and how sulforaphane (SFN), a novel promising nuclear factor-E2-related factor 2 (Nrf2) activator, exerted antioxidative stress through activating Nrf2 signaling.

Material/Methods: Cultured human trabecular meshwork cells (HTMCs) were treated with SFN for 6 hours after establishing the oxidative stress model by hydrogen peroxide (H_2O_2). The cell viability, the level of intercellular reactive oxygen species (ROS), and the apoptosis rate were observed using various kits. In addition, the gene and protein expression of Nrf2 and the phase II antioxidative enzymes were determined by performing qRT-PCR and western blotting.

Results: In H_2O_2-treated HTMCs, SFN protected HTMCs from oxidative stress damage and decreased the intracellular ROS accumulation, thus inhibiting cell apoptosis. SFN also increased the gene and protein expression of phase II antioxidative enzymes such as NAD(P)H: quinone oxidoreductase 1 (NQO-1), heme oxygenase-1 (HO-1), glutamate-cysteine ligase catalytic subunit (GCLC), and glutamate-cysteine ligase modifier subunit (GCLM) by Nrf2-dependent pathway. Furthermore, investigations of the pathway showed that HTMCs pretreated with LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K), downregulated the expression of phase II antioxidative enzymes, partly.

Conclusions: These results indicated a novel application for SFN in attenuating H_2O_2-induced oxidative stress in HTMCs through activating PI3K/Akt/Nrf2 signaling pathway.

MeSH Keywords: **Oxidative Stress • Phosphatidylinositol 3-Kinases • Proto-Oncogene Proteins c-akt**

Abbreviation: **Akt** – serine/threonine kinase; **AMD** – age-related macular degeneration; **ARE** – antioxidant response elements; **BSA** – bovine serum albumin; **DMEM** – Dulbecco's modified Eagle's medium; **FBS** – fetal bovine serum; **GCLC** – glutamate-cysteine ligase catalytic subunit; **GCLM** – glutamate-cysteine ligase modifier subunit; **HO-1** – heme oxygenase-1; **H_2O_2** – hydrogen peroxide; **HTMCs** – human trabecular meshwork cells; **IOP** – intraocular pressure; **Keap1** – Kelch-like associated protein 1; **NQO-1** – NAD(P)H: quinone oxidoreductase 1; **Nrf2** – nuclear factor-E2-related factor 2; **PI3K** – phosphatidylinositol 3-kinase; **POAG** – primary open-angle glaucoma; **PVDF** – polyvinylidene fluoride; **ROS** – reactive oxygen species; **ROP** – retinopathy of prematurity; **RPE** – rigment epithelial cells; **SD** – standard deviation; **SDS-PAGE** – sodium dodecyl sulfate-polyacrylamide gels for electrophoresis; **SFN** – sulforaphane

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Background

Glaucoma, the leading cause of irreversible blindness, is considered a progressive optic neuropathy, the most common form of which is primary open-angle glaucoma (POAG) [1]. Although the exact pathogenesis of POAG remains elusive, recent studies showed that oxidative stress played an important role in the progression of POAG [2,3]. On the one hand, oxidative stress promotes the production of reactive oxygen species (ROS), causing toxic reactions and oxidative damage [4]. On the other hand, oxidative stress is crucial to the degradation, dysfunction, and loss of trabecular meshwork cells (TMCs), leading to an elevated intraocular pressure (IOP), which is considered to be one of the important risk factors for POAG [3]. Therefore, recent studies have focused on the pathogenic of oxidative stress and potential anti-oxidative agents.

A number of protective mechanisms, including antioxidants and endogenous antioxidative enzymes, are initiated by the cells themselves to respond to antioxidative stress [5]. The antioxidative enzymes such as phase II antioxidative enzymes NAD(P)H: quinone oxidoreductase 1 (NQO-1), heme oxygenase-1 (HO-1), glutamate-cysteine ligase catalytic subunit (GCLC), and glutamate-cysteine ligase modifier subunit (GCLM) can be regulated by nuclear factor-E2-related factor 2 (Nrf2) signaling pathway [6]. Nrf2 is an essential transcription factor, and a specific receptor of which is Kelch-like associated protein 1 (Keap1) [6]. Activation of Nrf2 signaling pathway was shown to play an essential role in protecting against oxidative stress in many tissues including lung, liver, and brain [7,8] and alleviated oxidative damage in many ocular diseases, such as retinal ischemia-reperfusion injury [9], diabetic retinopathy [10], retinopathy of prematurity (ROP) [11], and age-related macular degeneration (AMD) [12].

Sulforaphane (SFN) is a natural dietary isothiocyanate found in cruciferous vegetables such as cabbage, Brussel sprouts, and broccoli [13]. SFN, as a novel promising Nrf2 activator, has attracted much attention because of its antioxidant effects. By sulfhydryl reaction with Keap1, SFN forms thioacyl adducts and advances the destruction of Nrf2-Keap1 interaction [14]. Sohel et al. reported that SFN protects granulosa cells against oxidative stress via activation of Nrf2/ARE (antioxidant response elements) pathway [15].

In this study, we investigated whether SFN might alleviate the oxidative stress damage induced by hydrogen peroxide (H_2O_2) in human trabecular meshwork cells (HTMCs) by activating Nrf2 signaling, and we explored the possible underlying mechanisms.

Material and Methods

Reagents

SFN and H_2O_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Cell culture reagents were offered from Gibco (Grand Island, NY, USA). Inhibitor of phosphatidylinositol 3-kinase (PI3k), LY294002, was purchased from PeproTech (NJ, USA).

Cell cultures

HTMCs were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in 8 mL Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 20% fetal bovine serum (FBS, Gibco). The medium was replaced every 3 days, and the cells were passaged when the cells reached 80% to 90% confluency. The cells of passage 3–5 were prepared for later experiments.

Cell viability assays

HTMC cells were added at 1×10^3 HTMCs/well to 96-well plates and cultured overnight. Then cells were treated with multiple concentrations of H_2O_2 (0, 1 μM, 10 μM, 100 μM, and 200 μM) for 24 hours with or without the pretreatment of SFN (0, 10 μM, 20 μM, and 30 μM). A Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used to determine the toxic effects of H_2O_2 and SFN on HTMCs according the manufacture's protocols. The absorbance value was recorded at 450 nm.

Intracellular ROS assays

HTMCs were inoculated into 6-well plates after the H_2O_2 induced oxidative stress model, and treated with SFN for 6 hours, washed 3 times with phosphate-buffered saline (PBS), then, the production of intracellular ROS was detected by Reactive Oxygen Species Assay Kit (Beyotime, Shanghai, China) and assayed by a microplate reader (Tecan Spark 10M, Switzerland) at 488 nm according to the manufacture's protocols.

Annexin V/FITC assay

HTMCs were inoculated into 6-well plates, after H_2O_2 and SFN treatment, according to the instruction of a FITC Annexin V apoptosis detection kit (BD Biosciences, Franklin, NJ, USA). After collected and resuspended in 100 μL buffer, cells were stained with Annexin V-FITC and propidium iodide (PI) for 15 minutes on ice. Then, cells were suspended in precooled buffer and analyzed by CytExpert software (Beckman-Coulter, Miami, FL, USA).

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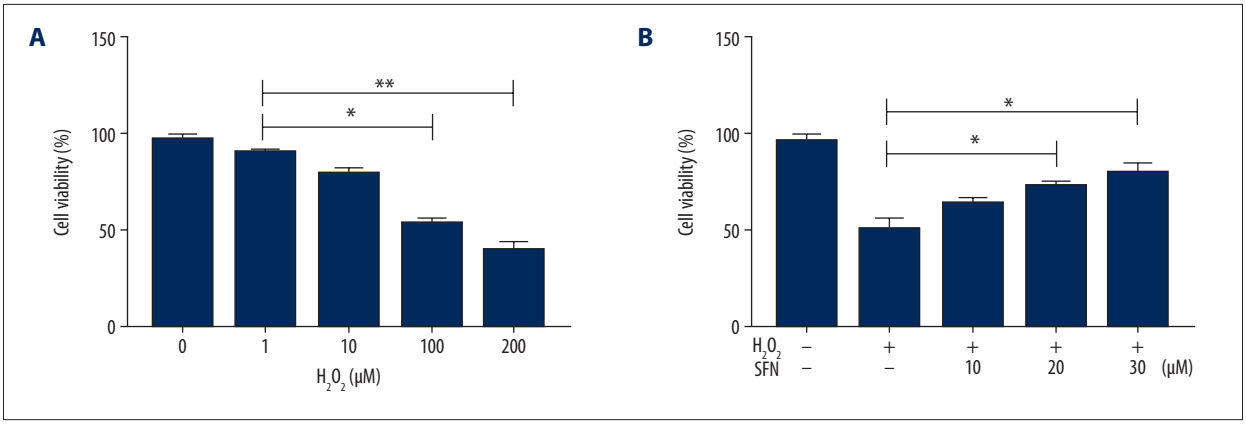

Figure 1. Sulforaphane (SFN) protects human trabecular meshwork cells (HTMCs) from H_2O_2-induced oxidative damage. HTMCs were treated with H_2O_2 (1 μM, 10 μM, 100 μM, and 200 μM) for 24 hours with or without pretreatment of SFN (10 μM, 20 μM, and 30 μM) for 6 hours. Cell viability were determined by using a CCK-8 (**A, B**). Data were expressed as mean ± standard deviation. * P<0.05, ** P<0.01. CCK-8 – Cell Counting Kit 8; H_2O_2 – hydrogen peroxide.

Quantitative real-time reverse transcriptase (qRT)-PCR

Total RNAs were isolated from the HTMCs using TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA, USA). RNA was reversed transcribed into cDNA by the PrimeScript RT Reagent kit (Takara, Shiga, Japan) which was used to perform the qRT-PCR analyses using the SYBR Premix ExTaqII (TliRNaseHPlus) kit (Takara) according to the manufacture's instruction. The primers used were as follows:
NOQ-1: forward,5'-AACCAACAGAGCCAATC-3',
reverse, 5'-CCTCCATCCTTTCCTC-3';
HO-1: forward,5'-CTGGCTTCCTTCCCTTGAG-3',
reverse, 5'-CTTTGGGTTGGAGATGT-3';
GCLM: forward,5'-AATCTTGCCTCCTGCTGTGT-3',
reverse, 5'-CTCGTGTGCTCGAATGTCAG-3';
GCLC: forward, 5'-AAGCCTCCTCCTCCAAACTC-3',
reverse, 5'-AGCACCACGAACACCACATA-3';
β-actin: forward, 5'-CTGTCCACCTTCCAGCAGA -3';
reverse, 5'-AGCCATGCCAATCTCATCTC-3'.

Quantitative and analysis of mRNA was performed by using Vii7 System (Applied Biosystems, Waltham, MA, USA).

Western blotting

Briefly, after treatment, the protein of the cytoplasm and nuclei was extracted by using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China) and concentrations were quantified by a BCA protein assay kit (Beyotime). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE) for 80 minutes, and then transferred onto a polyvinylidene fluoride (PVDF) membrane. The PDVF membranes were blocked in 5% bovine serum albumin (BSA) for 1 hour at room temperature, and incubated with rabbit anti-NQO-1, anti-HO-1, anti-GCLM, anti-GCLC, and anti-Nrf2, and mouse anti-GAPDH and anti-Lamin B primary antibodies at 4°C overnight, followed by secondary antibody goat anti-rabbit IgG and anti-mouse IgG for 1 hour at room temperature. The protein signals were observed using ECL reagent (Thermo Fisher, Rockford, IL, USA).

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Difference between 2 groups were analyzed by the Student's t-test, multiple group were assessed by one-way ANOVA using SPSS 22.0 (IBM Inc., Chicago, IL, USA). Statistical significance was established at P<0.05. The experiments were repeated 3 times.

Results

SFN protected HTMCs from H_2O_2-induced oxidative stress damage and decreased the intracellular ROS production

We exposed H_2O_2 to HTMCs to induce obvious oxidative injury, since this model has been widely used in previous studies [16,17]. First, HTMCs were treated with H_2O_2 at different concentrations (1 μM, 10 μM, 100 μM, and 200 μM) for 24 hours. The results of CCK-8 showed that H_2O_2 decreased the cell viability of HTMCs, and there was a significant reduction in cell viability at 100 μM of H_2O_2 (P<0.01). In addition, we found that H_2O_2 (100 μM) displayed about 50% cytotoxicity in HTMCs (Figure 1A). Thus, 100 μM was determined as the optimal concentration for follow-up experiments. Next, HTMCs were treated with various concentrations (10 μM, 20 μM, and 30 μM) of SFN for 6 hours, followed by the stimulation of H_2O_2 (100 μM) for 24 hours. As showed in Figure 1B, SFN increased cell viability in a dose-dependent manner and SFN (20 μM and 30 μM) suppressed H_2O_2-induced cytotoxicity significantly (P<0.05).

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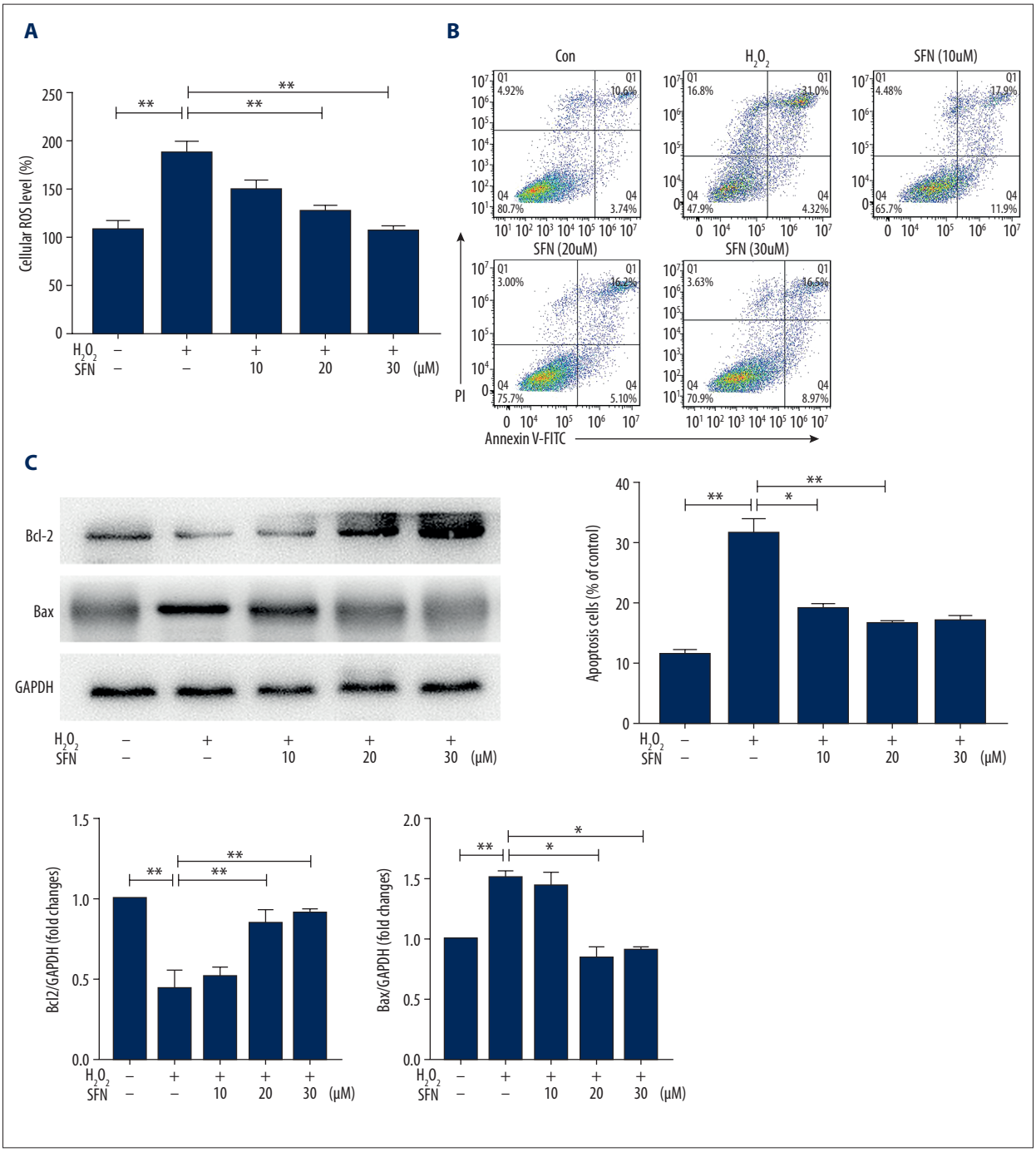

Figure 2. Sulforaphane (SFN) inhibited H_2O_2-induced ROS over expression and apoptosis in human trabecular meshwork cells (HTMCs). HTMCs were treated with H_2O_2 (1 μM, 10 μM, 100 μM, and 200 μM) for 24 hours with or without pretreatment of SFN (10 μM, 20 μM, and 30 μM) for 6 hours. (**A**) The levels of intercellular ROS were detected by using a ROS assay kit. (**B**) Apoptotic cells were tested by a FITC Annexin V apoptosis detection kit. (**C**) Protein expression of PARP, Bcl2, and Bax. Data were expressed as mean ± standard deviation. * $P<0.05$, ** $P<0.01$. H_2O_2 – hydrogen peroxide; ROS – reactive oxygen species

Then, we investigated the effects of H_2O_2 and SFN on the production of ROS, which was a marker of oxidative stress injury. Our findings suggested that treatment with H_2O_2 alone increased intracellular ROS levels obviously ($P<0.01$), but were blocked by pretreatment with SFN. Furthermore, SFN (20 μM and 30 μM) was significant for reducing the production of intracellular ROS (Figure 2A).

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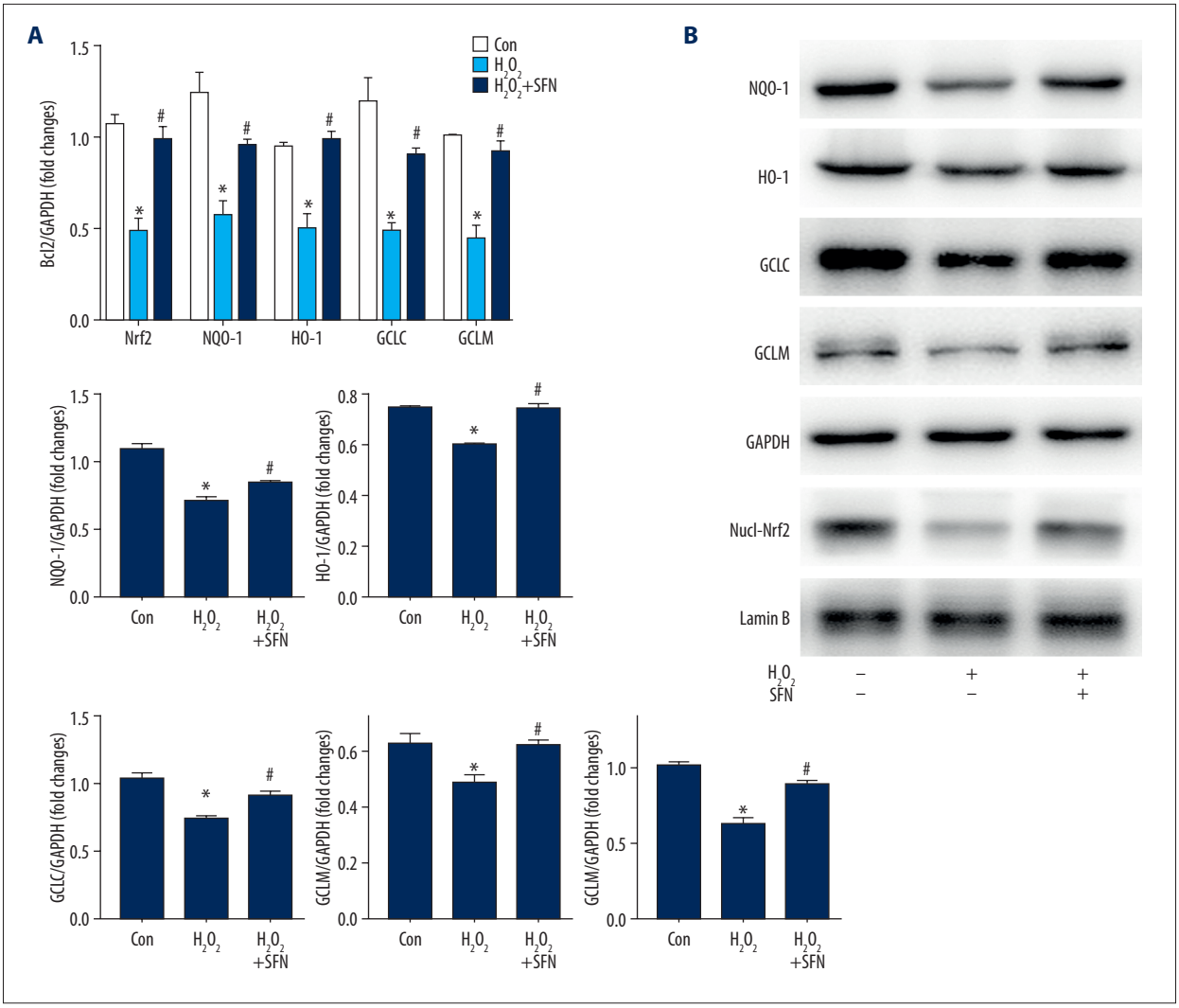

Figure 3. Sulforaphane (SFN) activates Nrf2 signaling in human trabecular meshwork cells (HTMCs). HTMCs were treated with SFN (20 μM) for 6 hours, followed by the stimulation of H_2O_2 (100 μM) for 24 hours. mRNA levels and protein expression of Nrf2 and phase II antioxidative enzymes NQO-1, HO-1, GCLC, and GCLM (**A, B**). Data were expressed as mean ± standard deviation. * $P<0.05$, Control versus H_2O_2, # $P<0.05$, H_2O_2 versus H_2O_2+SFN. Nrf2 – nuclearfactor-E2-related factor 2; NQO-1 – NAD(P)H: quinone oxidoreductase 1; HO-1 – heme oxygenase-1; GCLC – glutamate-cysteine ligase catalytic subunit; GCLM – glutamate-cysteine ligase modifier subunit; H_2O_2 – hydrogen peroxide.

It has been demonstrated that a high level of intracellular ROS in cells induces apoptosis. Thus, we examined the effect of SFN on apoptosis. As shown in Figure 2B, H_2O_2 increased the expression of apoptotic protein Bcl2 and PARP, which inhibited the expression of Bax, which significantly induced the occurrence of apoptosis. Compared with the H_2O_2 group, the apoptosis rate of cells with added SFN decreased, and the difference was significant when the concentration of SFN was 20 μM and 30 μM (Figure 2C). Therefore, we chose 20 μM as intervention concentration of SFN in subsequent experiments.

SFN induced phase II antioxidative enzymes expression by activating Nrf2 signaling

A previous study clarified that SFN induced phase II antioxidative enzymes expression in many cells, such as alveolar macrophages [18], primary rat hepatocytes [19], and retinal pigment epithelium [20]. To investigate whether SFN also induced the expression of phase II antioxidative enzymes in HTMCs, we determined the mRNA and protein expression of these enzymes by performing qRT-PCR and western blot analyses. As displayed in the Figure 3A and 3B, H_2O_2 alone inhibited the mRNA and protein expression of Nrf2, NQO-1, HO-1, GLCL, and GCLM, but pretreatment with SFN suppressed the

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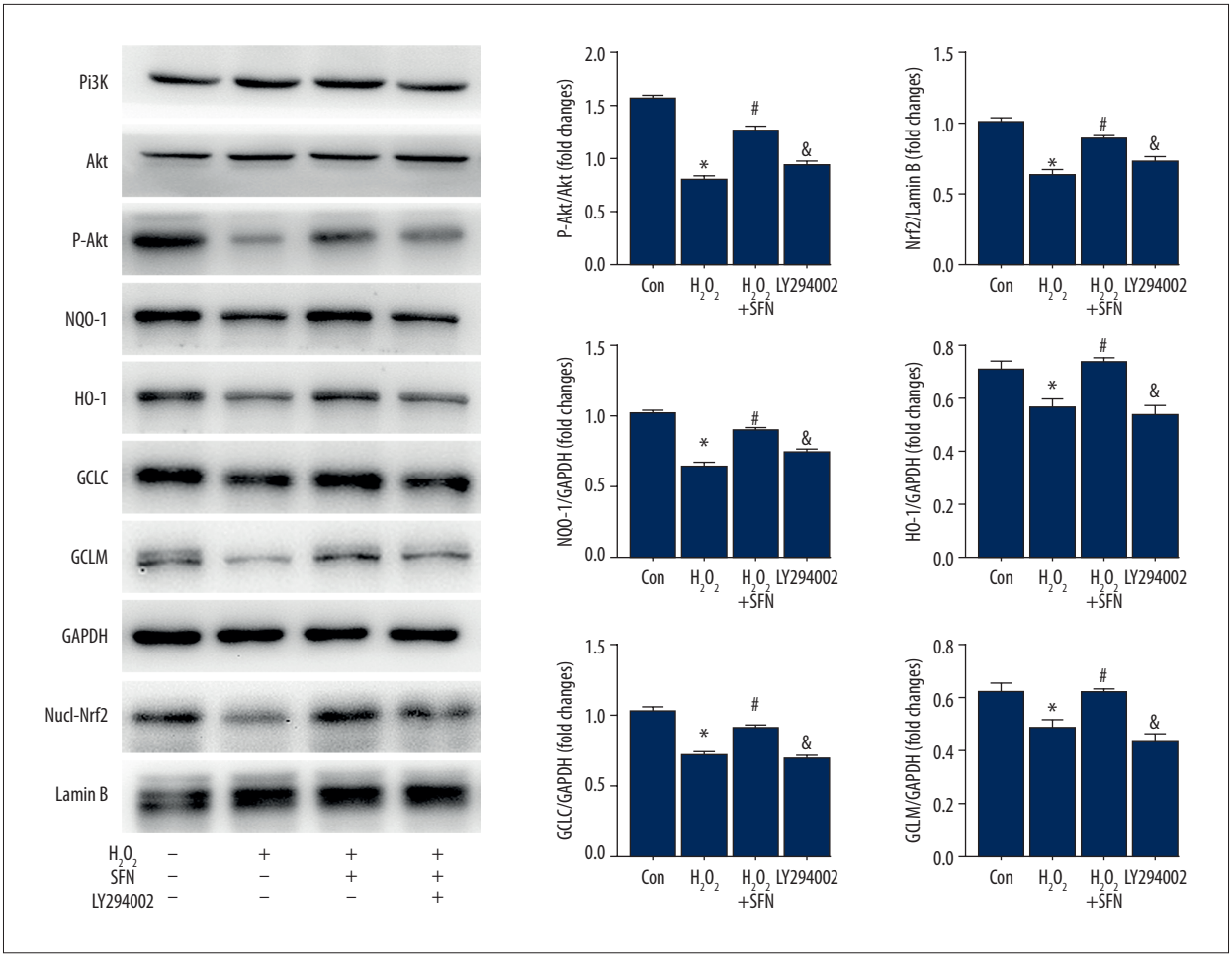

Figure 4. Sulforaphane (SFN) exerts an anti-oxidative role by PI3K/Akt-mediated Nrf2 signaling pathway. Human trabecular meshwork cells (HTMCs) were pretreated with LY294002 (20 μM) for 6 hours prior to being exposed to SFN (20 μM) for 6 hours and H_2O_2 (100 μM) for 24 hours respectively. Protein expression of PI3K, Akt, P-Akt, Nrf2, and phase II antioxidative enzymes NQO-1, HO-1, GCLC, and GCLM. Data were expressed as mean ± standard deviation. * $P<0.05$, Control versus H_2O_2, # $P<0.05$, H_2O_2 versus H_2O_2+SFN, & $P<0.05$, H_2O_2+SFN versus LY294002. PI3K – phosphatidylinositol 3-kinase; Akt – serine/threonine kinase; Nrf2 – nuclearfactor-E2-related factor 2; H_2O_2 – hydrogen peroxide; NQO-1 – NAD(P)H: quinone oxidoreductase 1; HO-1 – heme oxygenase-1; GCLC – glutamate-cysteine ligase catalytic subunit; GCLM – glutamate-cysteine ligase modifier subunit.

decreasing effects of H_2O_2 on the secretion of phase II antioxidative enzymes in HTMCs.

SFN activated Nrf2 signaling via PI3K/Akt pathway

Next, we investigated the upstream signaling pathway involved in SFN-mediated Nrf2 signaling activation. Since the PI3K/Akt pathway has been reported to play a major role in modulating Nrf2 signaling activation, we examined the protein expression of the PI3K/Akt pathway and found that SFN remarkably promoted the phosphorylation of Akt (Figure 4). To verify that SFN activated Nrf2 signaling via the PI3K/Akt pathway, HTMCs were exposed to the inhibitor of PI3K, LY294002 (20 μM) for 2 hours. The results demonstrated that after the addition of LY294002, phosphorylation of Akt decreased, thereby reducing the protein expression of Nrf2 and phase II antioxidative enzymes, which reversed the activation of SFN to Nrf2 signaling.

Discussion

In the present study, we found that different concentrations of H_2O_2 could decrease cell viability of HTMCs to varying degrees, and SFN displayed a dose-dependent response in increasing cell viability of H_2O_2-treated HTMCs. In addition, SFN inhibited the production of intracellular ROS induced by H_2O_2 in HTMCs, thereby restraining apoptosis. SFN activated Nrf2 signaling, promoting the gene and protein expression of phase II antioxidative enzymes

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NOQ-1, HO-1, GCLC, and GCLM. Further studies suggested that the upregulating effects on the expression of phase II antioxidative enzymes were partially suppressed after pretreating with LY294002, an inhibitor of PI3k. These results indicated that SFN protected HTMCs from H_2O_2-induced oxidative damage by PI3K/Akt-mediated Nrf2 signaling pathway activation.

POAG is a chronic, irreversible optic neuropathy. Although the exact pathogenesis of POAG is still not clear, a variety of pathogenic theories have proposed for the development of this disease, such as elevated IOP, optic nerve susceptibility, vascular disorders, and oxidative stress [21]. Most studies focused on glaucoma retinopathy, which occurs at the terminal disease stage [22]. Sacca et al. [4] demonstrated that ROS over-production induced by oxidative stress contributed to retinal degeneration by inducing lipid peroxidation, calcium overload, and DNA damage, eventually causing retinal cell death and apoptosis. The relationship between increased IOP caused by trabecular meshwork dysfunction and POAG progression is attracting increasing attention. The TMCs is a complex tissue with different cell types, morphology and function of which contribute to maintaining normal IOP [23]. Any factors that affect the function of TMCs may lead to increasing IOP, promoting the progression of POAG. The oxidative denaturation of trabecular meshwork causes damage to the outflow tract and regional blood perfusion [24]. In early glaucoma, mitochondrial damage and endothelial dysfunction caused by oxidative stress promotes endogenous cell loss and defense function reduction, thus activating the apoptosis of TMCs [2].

Nrf2 is a transcription factor that controlling hundreds of detoxifying and antioxidant genes [25]. When exposed to oxidation or electrophilic stress, the Nrf2-Keap1 complex in the cytoplasm will be separated. Then Nrf2 translocates into the nucleus where it combines with antioxidant response elements to initiate the gene expression regulation of antioxidative enzymes system, thereby enhancing cell resistance to oxidative stress and nucleophilic compounds [26,27]. The activation of Nrf2 has been implicated as an important protective role in protecting against oxidative stress in multiple cells [28,29]. Harvey et al. [18] reported that activating Nrf2 signaling pathway inhibited oxidative stress caused by cigarette smoke, leading to attenuating chronic obstructive pulmonary disease in mice. Exerting antioxidation by activating Nrf2 signaling is mainly dependent on the release of antioxidant kinase, such as NQO-1, HO-1, GCLC, and GCLM. Ross et al. [30] found that NQO-1 protected cell membranes from oxidative damage by decreasing endogenous quinine compounds. HO-1 is a major antioxidant enzyme that catalyzes the speed limiting step of heme catabolism, leading to the formation of bilirubin, free iron and carbon monoxide, thus increasing the resistance of cells to oxidative damage [31]. Glutamate cysteine ligase (GCL), is a heterodimeric protein composed of catalytic (GCLC) and modifier (GCLM) subunits. Studies have shown that GCL is the main

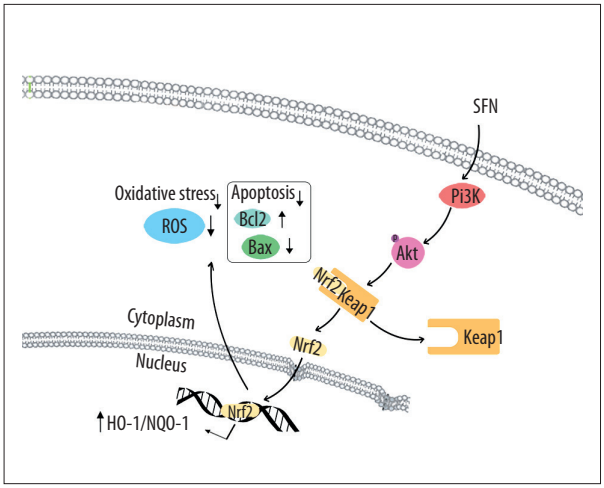

Figure 5. Sulforaphane (SFN) exerts antioxidant stress through PI3K/Akt and Nrf2 signaling pathways. SFN, as a new type of Nrf2 agonist, activate Nrf2 by promoting PI3K/Akt signaling pathways, mainly enhancing the phosphorylation of Akt. Nrf2 is translocated into nuclei after released from Keap1 to induce the transcription of phase II antioxidative enzymes NQO-1, HO-1, GCLC, and GCLM. However, the direct relationship between PI3K/Akt and Nrf2 requires future researches to elaborate. Nrf2 – nuclearfactor-E2-related factor 2; PI3K – phosphatidylinositol 3-kinase; Akt – serine/threonine kinase; NQO-1 – NAD(P)H: quinone oxidoreductase 1; HO-1 – heme oxygenase-1; GCLC – glutamate-cysteine ligase catalytic subunit; GCLM – glutamate-cysteine ligase modifier subunit.

determinant of cellular GSH level, effecting the antioxidant capacity of cells [32]. This study is the first to clarify the activation of Nrf2 signaling pathways in HTMCs, which could also increase the expression of antioxidant kinase NQO-1, HO-1, and GCL, playing a role of antioxidant stress.

Nrf2 signaling can be activated by many agents, most of which are plant-chemical extracts or their derivatives, such as SFN [19], carotenoids [29], and flavonoids [33]. Unlike other antioxidants, SFN does not scavenge reactive oxygen species directly, but relies on the ability to induce the expression of antioxidant enzymes [34]. Gao et al. [14] showed that SFN protected retinal pigment epithelial cells (RPE) from photooxidative stress via Nrf2 signaling. Sohel et al. [15] reported that SFN promoted the release of NQO-1, HO-1 via Nrf2/antioxidant response elements pathway. The present study also revealed that SFN inhibited H_2O_2-induced intracellular ROS production and increased the HTMCs activity.

PI3K/Akt pathway signaling plays an important role in modulating Nrf2 signaling [29]. It has been reported that drugs such as astaxanthin activated Nrf2 signals in many cells, and the action was partly dependent on the activation of the PI3K/Akt pathway [28,35,36].

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Conclusions

Consistent with previous studies, we found that HTMCs pretreated with LY294002, an inhibitor of PI3K. suppressed expression of antioxidant enzymes increased by SFN partially.

In summary, we suggest that SFN protects HTMCs from H_2O_2-induced oxidative stress via the PI3K/Akt-mediated Nrf2 pathway (Figure 5). Our study establishes a basis for further studies on SFN as a promising trabecular meshwork cytoprotective agent against oxidative stress.

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