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*Original Research*

# Syringic acid protects retinal ganglion cells against $H_2O_2$-induced apoptosis through the activation of PI3K/Akt signaling pathway

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**Abstract:** Oxidative damage is believed to contribute to the pathogenesis of diabetic retinopathy. Syringic acid (SA), a naturally occurring O-methylated trihydroxybenzoic acid monomer extracted from *Herba dendrobii*, has been shown to possess antioxidant activity. However, the effects of SA on apoptosis of retinal ganglion cells (RGCs) induced oxidative damages have not yet been explored. The present study aimed to detect the effects of SA against hydrogen peroxide ($H_2O_2$)-induced cell damage in RGCs and to investigate the molecular mechanisms involved in this process. In the present study, we revealed that SA pretreatment obviously inhibited $H_2O_2$-induced RGC-5 cell injury. SA pretreatment also decreased $H_2O_2$-induced ROS production and MDA content in RGC-5 cells. In addition, SA pretreatment increased Bcl-2 expression and decreased the expression of Bax and cleaved caspase-3 in $H_2O_2$-induced RGC-5 cells. Moreover, SA pretreatment obviously increased PI3K and Akt phosphorylation in $H_2O_2$-induced RGC-5 cells. In conclusion, our results suggest that SA may protect RGC-5 cells against apoptosis induced by $H_2O_2$ through the activation of PI3K/Akt signaling pathway. Thus, SA may be beneficial in the treatment of diabetic retinopathy.

***Key words:*** Syringic acid (SA), diabetic retinopathy, retinal ganglion cells (RGCs), apoptosis.

## Introduction

Diabetic retinopathy, one of the leading causes of visual impairment among aged adults in the world, is characterized by the loss of retinal ganglion cells (RGCs) (1, 2). Although reduction in hyperglycemia has been shown to exert positive effects on the development and progression of diabetic retinopathy, the pathogenesis of diabetic retinopathy remains to be elucidated. Thus, it is necessary to develop new therapeutic strategies for this disease. Oxidative damage is believed to contribute to the pathogenesis of diabetic retinopathy. The retina is highly vulnerable to oxidative injury induced by reactive oxygen species (ROS) (3). Recent studies have shown that increased oxidative stress induces the dysfunction of signaling pathways in RGCs, thereby resulting in RGCs injury (4-6).

*Herba dendrobii* is found in the stem of the orchid Dendrobium nobile Lindl and in many other orchid species of the *Dendrobium* genus (7). Syringic acid (SA), a naturally occurring O-methylated trihydroxybenzoic acid monomer extracted from *Herba dendrobii*, has been shown to possess antioxidant, anti-tumor, and antimicrobial activities. Most recently, one study showed that SA reduced oxidative stress and neuron apoptosis in spinal cord ischemia/reperfusion rats (8). In addition, Wei *et al*. confirmed that SA is capable of protecting rats from developing experimentally induced diabetes cataracts both *in vitro* and *in vivo* (9). However, the effects of SA on apoptosis of RGCs induced oxidative damages have not yet been explored. The present study aimed to detect the effects of SA against hydrogen peroxide ($H_2O_2$)-induced cell damage in RGCs and to investigate the molecular mechanisms involved in this process. Our results showed that SA protects RGCs from apoptosis induced by $H_2O_2$ through the activation of PI3K/Akt signaling pathway.

## Materials and Methods

### RGC-5 cell culture and treatment

RGC-5 cells were purchased from the Centre of Cells Resource, Shanghai Institute of Life Science, Chinese Academy of Sciences (China) and cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma; St. Louis, MO, USA). All cells were maintained at 37°C in a humidified 5% $CO_2$, 95% air incubator. To induce oxidative stress, RGC-5 cells at a density of $1\times10^4$ cells/well were seeded into 96-well and cultured for 24 h. They were then pretreated with various concentrations (10, 20 and 40 μM) of SA (Sigma, St. Louis, MO, USA) for 24 h prior to addition of 250 μM $H_2O_2$ for 24 h.

### Cell viability assay

Cell viability was measured with a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

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In brief, RGC-5 cells were pre-treated with various concentrations of SA (10, 20 and 40 μM) for 24 h prior to exposure with 250 μM $H_2O_2$ for 24 h. CCK-8 solution (10 μl) was added to each well and the plates were incubated for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA).

**Cell cytotoxicity assay**

Cell cytotoxicity was evaluated by the lactate dehydrogenase (LDH) assay using the cytotoxicity detection kit in accordance with the manufacturer's instructions. Briefly, RGC-5 cells were treated with different concentrations of SA (10, 20 and 40 μM) for 24 h. Then, the media were collected and centrifuged at 10,000×g for 3 min at 4 °C, and the supernatants were harvested. One hundred microliters of the LDH reaction was loaded into each well and incubated at room temperature in the dark for 30 min. The absorbance of each solution at 490 nm was measured using a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA).

**Measurement of Intracellular ROS generation and MDA**

ROS level was evaluated using 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) (Sigma–Aldrich, St. Louis, MO, USA). Treated cells were washed twice with PBS, and then incubated with 10 mM DCFH-DA for 30 min at 37 °C in the dark. The fluorescence from the DCF was analyzed using a high content screening system (ArrayScanVTI, Thermo Fisher Scientific, Walsam, MA, USA) with the excitation wavelength set at 488 nm and the emission wavelength set at 525 nm.

The level of MDA was measured in RGC-5 cells using a MDA detection kit (Beyotime Institute of Biotechnology, Nanjing, China).

**Western blot**

The proteins were extracted from RGC-5 cells using RIPA lysis buffer (Beyotime, Nantong, China). The protein concentration in the lysates was determined using a BCA protein assay kit (Beyotime, Nantong, China). Samples (40 μg) were subjected to 10% SDS-PAGE gel and transferred to Immobilon-P Transfer Membranes (Millipore). The membranes were incubated overnight at 4 °C with one of the following primary antibodies: anti-Bax (1:1000; Cell Signaling Technology, Beverly, MA, USA), anti-Bcl-2 (1:1000; Cell Signaling Technology), anti-cleaved caspase-3 (1:1000; Cell Signaling Technology), anti-p-PI3K antibody (1:1000; Cell Signaling Technology), anti-PI3K antibody (1:1000; Cell Signaling Technology), anti-p-Akt antibody (1:1000; Cell Signaling Technology), anti-Akt antibody (1:1000; Cell Signaling Technology) and anti-β-actin (1:2000; Santa Cruz Biotechnology). Subsequently, the membranes were incubated with peroxidase-conjugated secondary antibody at room temperature for 1 h. The bands were visualized with Luminescent image analyzer (ImageQuant LAS 4000, GE Healthcare, UK) using chemiluminescence (GE Healthcare, Buckinghamshire, UK).

**Statistical analysis**

Results from at least three independent experiments were expressed as mean ± SD. Statistical significance was analyzed with the one-way factorial ANOVA or the Student's two-tailed t-test. Differences with $P<0.05$ were considered statistically significant.

## Results

**SA protects RGC-5 cells from $H_2O_2$-induced insults**

First, we investigated the effect of SA on $H_2O_2$-induced RGC-5 cells viability using the CCK-8 assay. As shown in Figure 1A, compared with untreated RGC-5 cells, treatment with SA alone did not obviously affect cell viability. When RGC-5 cells were treated with $H_2O_2$ for 24 h, the cell viability was significantly decreased. However, pretreatment with SA reversed this effect, exhibiting a dose-dependent manner (Figure 1B).

We further examined whether SA pretreatment could influence $H_2O_2$-induced RGC-5 cells cytotoxicity. The results showed that $H_2O_2$ treatment significantly increased LDH release, however, pretreatment with SA significantly reduced the release of LDH in RGC-5 cells (Figure 1C).

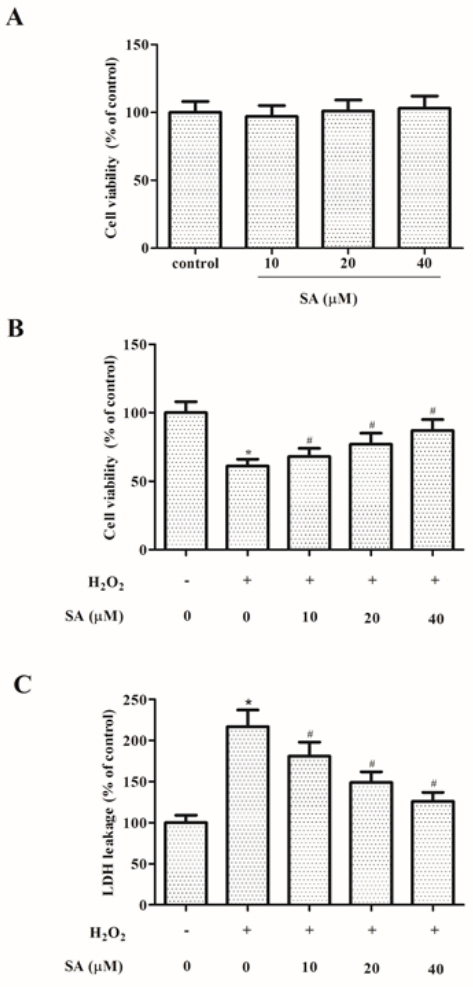


**Figure 1.** SA protects RGC-5 cells from $H_2O_2$-induced insults. RGC-5 cells were treated with SA (10, 20 and 40 μM) for 24 h, and cell viability was determined by the CCK-8 assay. RGC-5 cells were treated with SA (10, 20 and 40 μM) for 24 h prior to $H_2O_2$ insult and then incubated for 24 h. (B) Cell viability was determined by the CCK-8 assay; (C) LDH leakage was determined using a commercial LDH kit. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* $H_2O_2$ group.

### SA inhibits ROS production and lipid peroxidation induced by $H_2O_2$ in RGC-5 cells

We next determined the effects of SA on the $H_2O_2$-induced oxidative stress in RGC-5 cells. As shown in Figure 2A, OGD exposure markedly increased cellular intracellular ROS generation, while SA (10, 20 and 40 μM) pretreatment decreased $H_2O_2$-induced ROS production in RGC-5 cells, exhibiting a dose-dependent manner. Similarly, SA pretreatment resulted in a significant decrease in $H_2O_2$-induced MDA content in a dose-dependent manner (Figure 2B).

### SA protects RGC-5 cells against apoptosis induced by $H_2O_2$

Oxidative stress-induced RGCs apoptosis is involved in the progression of diabetic retinopathy. Therefore, we investigated the effect of SA on RGC-5 cells apoptosis induced by $H_2O_2$. As expected, Western blot analysis showed that $H_2O_2$ significantly increased the expression levels of Bax and caspase-3 proteins, and decreased Bcl-2 expression; while SA pretreatment down-regulated the expression of Bax and caspase-3, and increased the expression of Bcl-2 in RGC-5 cells (Figure 3).

### SA attenuates $H_2O_2$-induced impairments to RGC-5 cells by the activation of the PI3K/Akt signaling pathway

Activation of PI3K/Akt signaling pathway has been reported to participate in RGCs apoptosis induced by $H_2O_2$. So, to explore the molecular mechanism by which

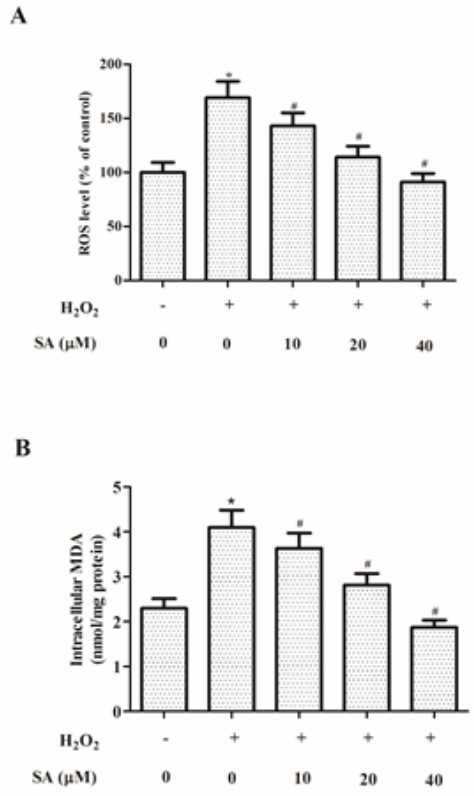


**Figure 2.** SA inhibits ROS production and lipid peroxidation induced by $H_2O_2$ in RGC-5 cells. RGC-5 cells were treated with SA (10, 20 and 40 μM) for 24 h prior to $H_2O_2$ insult and then incubated for 24 h. (A) The level of ROS was measured by incubating with DCFH-DA dye and analyzed by flow cytometry. (B) Intracellular MDA was detected by thiobarbituric acid colorimetric assay. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* $H_2O_2$ group.

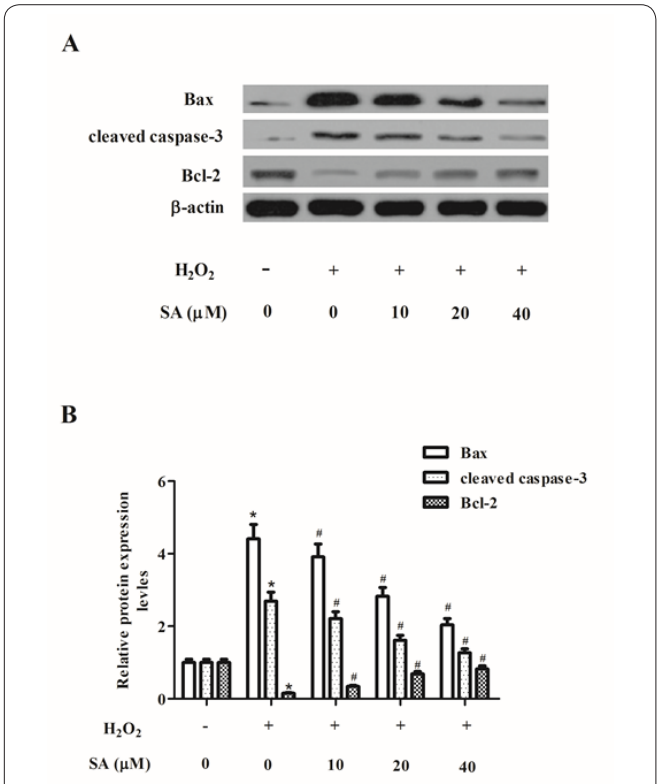


**Figure 3.** SA protects RGC-5 cells against apoptosis induced by $H_2O_2$. RGC-5 cells were treated with SA (10, 20 and 40 μM) for 24 h prior to $H_2O_2$ insult and then incubated for 24 h. (A) The expression of Bcl-2, Bax, cleaved caspase-3, and β-actin proteins was detected by western blotting and representative blots were shown. (B) Quantification analysis of Bcl-2, Bax and cleaved caspase-3. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* $H_2O_2$ group.

SA inhibits $H_2O_2$-induced RGCs apoptosis, we investigated the protein expression levels of p-PI3K, PI3K, p-Akt and Akt by western blot. As shown in Figure 4, $H_2O_2$ markedly inhibited PI3K and Akt phosphorylation. However, SA pretreatment obviously increased PI3K and Akt phosphorylation in $H_2O_2$-induced RGC-5 cells.

## Discussion

In the present study, we revealed that SA pretreatment obviously inhibited $H_2O_2$-induced RGC-5 cell injury. SA pretreatment also decreased $H_2O_2$-induced ROS production and MDA content in RGC-5 cells. In addition, SA pretreatment increased Bcl-2 expression and decreased the expression levels of Bax and cleaved caspase-3 in $H_2O_2$-induced RGC-5 cells. Moreover, SA pretreatment obviously increased PI3K and Akt phosphorylation in $H_2O_2$-induced RGC-5 cells.

$H_2O_2$ is an exogenous source for ROS production and excess $H_2O_2$ can enter the cells and induce cytotoxicity due to its high membrane permeability , and has been reported to trigger apoptosis in RGC-5 cells (10). In this study, we used $H_2O_2$ as an exogenous inducer of oxidative stress in RGC-5 cells. As expected, we found that $H_2O_2$ significantly decreased RGC-5 cell viability. However, pretreatment with SA reversed this effect, exhibiting a dose-dependent manner.

Oxidative stress plays an important role in the pathogenesis of diabetic retinopathy (11-13). The production of ROS may cause the raise of oxidative stress

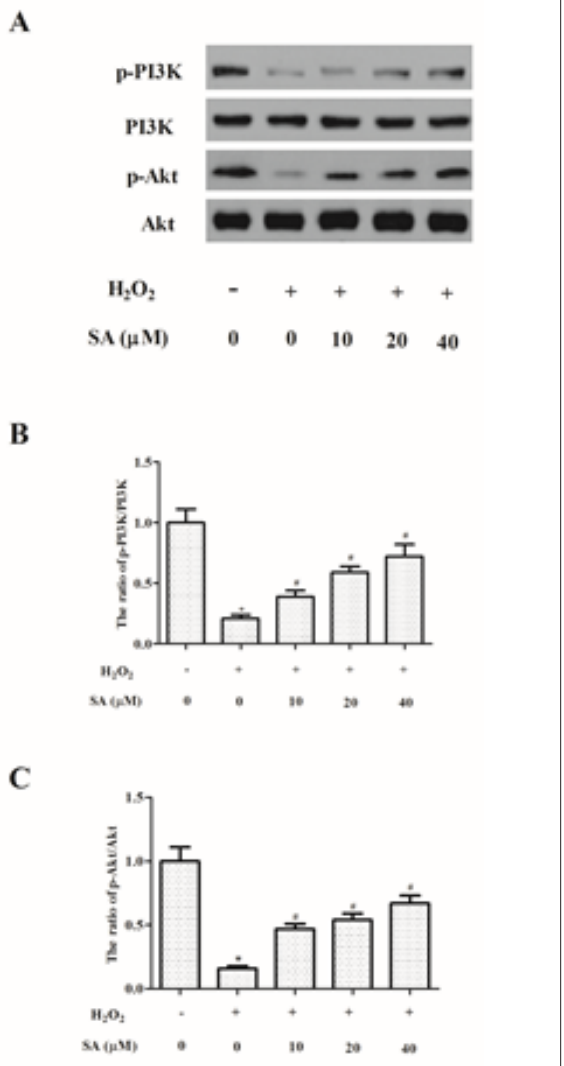


**Figure 1.** SA attenuates $H_2O_2$-induced impairments to RGC-5 cells by the activation of the PI3K/Akt signaling pathway. RGC-5 cells were treated with SA (10, 20 and 40 μM) for 24 h prior to $H_2O_2$ insult and then incubated for 24 h. (A) The expression of p-PI3K, PI3K, p-Akt and Akt proteins was detected by western blotting and representative blots were shown. (B) The optical density is expressed as the ratio of p-PI3K to PI3K; (C) The optical density is expressed as the ratio of p-Akt to Akt. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* $H_2O_2$ group.

which can damage the cellular organelle (14). Previous studies reported that RGCs death is caused by over production of ROS which may lead to diabetic retinopathy (15, 16). In this study, we observed that SA pretreatment significantly decreased the production of ROS induced by $H_2O_2$ in RGC-5 cells. MDA is a decomposition product of peroxidized polyunsaturated fatty acids, mainly considered as an index of lipid peroxidation (17). Morton *et al*. reported that SA is a strong inhibitor of low-density lipoprotein oxidation, contributing to the scavenging of free radicals, reducing production of MDA, and thus slowing atherosclerosis (18). Similarly, in this study, we found that SA pretreatment greatly reduced MDA content induced by $H_2O_2$ in RGC-5 cells. All those findings supported that SA protected RGCs from $H_2O_2$-induced cytotoxicity due to its radical scavenging activities.

Multiple lines of evidence showed that members of the Bcl-2 gene family play important roles in the activation and control of RGCs death (19-22). Caspases are key mediators of cell death and caspase-3 is an executioner for the death program in various cells in response to oxidant. Oxidative stress induces caspase-3 activation in RGCs (2, 23, 24). Notably, one study showed that SA significantly decreased the number of apoptotic neurons in rats with spinal cord ischemia/reperfusion (8). In line with the previous study, herein, our data revealed that SA pretreatment increased Bcl-2 expression and decreased the expression of Bax and cleaved caspase-3 in $H_2O_2$-induced RGC-5 cells. These results suggest that SA suppressed cell apoptosis by promoting the expression of Bcl-2 while inhibiting that of Bax and caspase-3 in RGC-5 cells induced by $H_2O_2$.

Recent studies have shown that the PI3K/Akt signaling pathway is important for RGC survival (25-27). Akt is a serine-threonine kinase that is activated by the secondary messenger PI3K. The up-regulation of p-Akt suppressed RGCs apoptosis via several mechanisms including alteration of gene expression, inhibition of caspase-3 activation and suppression of cytochrome c release from the mitochondria (28). Intravitreous administration of BDNF and IGF-1 induced the activation of Akt in retinas of optic nerve axotomized eyes and prevented RGC death (29, 30). Herein, we observed that SA pretreatment obviously increased PI3K and Akt phosphorylation in $H_2O_2$-induced RGC-5 cells. All these data suggest that SA inhibited the apoptosis of RGC-5 cells from $H_2O_2$-induced injury via the activation of PI3K/Akt signaling pathway.

In conclusion, our results demonstrate that SA may protect RGC-5 cells against apoptosis induced by $H_2O_2$ through the activation of PI3K/Akt signaling pathway. Thus, SA may be beneficial in the treatment of diabetic retinopathy.

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