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Ginsenoside Rg1 defenses PC-12 cells against hydrogen peroxide-caused damage via up-regulation of miR-216a-5p

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ABSTRACT

Background: Spinal cord injury (SCI) is a destructive trauma accompanied with local injury. Ginsenoside Rg1 exerts anti-apoptosis and anti-autophagy properties. Our goal was to study the protective mechanism of Rg1 in attenuating cell injury.

Methods: MiR-216a-5p inhibitor was transfected into PC-12 cells to verify the growth promoting roles of miR-216a-5p, then cells were pre-treated by Rg1 for 24 h and treated by 300 μM hydrogen peroxide (H_2O_2) for 1 h. Cell viability and apoptosis were tested through Cell Counting Kit-8 (CCK-8) and flow cytometry, respectively. Expression of miR-216a-5p and cell damage relative factors was tested via qRT-PCR and Western blot experiments.

Results: H_2O_2 induced cell activity suppression, apoptosis and clear autophagy well at the concentration of 300 μM Rg1 attenuated H_2O_2-induced cell injury at the concentration of 200 μM that it elevated cell activity, attenuated apoptosis and autophagy and activated phosphatidylinositol 3 kinase (PI3K)/AMP-activated protein kinase (AKT) and AMP-activated protein kinase (AMPK) signal pathways. Further, miR-216a-5p was up-regulated by Rg1.

Conclusion: Our study demonstrated that Rg1 attenuated H_2O_2-caused cell injury through positively regulated miR-216a-5p.

1. Introduction

Spinal cord injury (SCI) is a common and destructive trauma [1], mainly caused by external forces [2]. Due to limited therapy selections, more than 60% of damages occur at the cervical level [3], and lifetime care costs are about at $1.1-$4.2 million per patient [4]. SCI involves two different stages of tissue injury, called primary and secondary hurt [5]. In SCI, oxidative stress is considered to be an important cause of secondary damage progression [6], leading to cell apoptosis, autophagy and loss of neurological manifestation. So, reducing the apoptosis and autophagy to inhibit oxidative stress can effectively improve SCI and slow down the progression of secondary injury. Our study aimed to explore a safe and effective method for attenuating oxidative stress injury.

Ginsenosides is considered as one of the main pharmacological active ingredients of ginseng. It is a steroid compound [7]. Ginsenoside contains the Panaxatriol (Rg1, Rg2, Re and Rf) and Panaxadiol (Rb1, Rb2, Rc and Rd) classes [8]. Many beneficial effects of Rg1 have been proved in disorders such as hypertension [9], hypoxia/reoxygenation [10], Alzheimer's disease [11] *etc*. Importantly, it has been reported that Rg1 exerts roles in inhibiting cell apoptosis, thereby exhibiting notable cardioprotective effects on I/R damage through a variety of mechanisms [12]. Besides, Rg1 counteracts the aging of endothelial progenitor cells [13] and human fibroblasts [14] and exerts a notable role in suppressing autophagy in cardiomyocytes and renal tubular cells [15]. The influence of Rg1 in oxidative stress injury after SCI is unknown.

MicroRNAs (miRNAs) are involved in many biological processes with 22 nucleotides in length [16]. MiR-216a-5p, known as an oncogene presented as promoting tumor progression, is involved in the progression of many cancer subtypes [17]. It elevates cell proliferation, activity and motility, and inhibits apoptosis [17], indicating that miR-216a-5p has a positive effect on cell viability and anti-apoptosis, implying the growth promoting effect of miR-216a-5p. So it will be interesting to investigate, if there exerts regulation relation between miR-216a-5p and Rg1 in cell injury after SCI. Hydrogen peroxide (H_2O_2) treatment was often employed to establish cellular model of SCI [18–21]. Based on the above questions and evidences, we are

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presenting new mechanism of Rg1 in attenuating hydrogen peroxide (H_2O_2)-caused cell damage in PC-12 cells.

2. Materials and methods

2.1. Cell

PC-12 cells were bought from Kunming Institute of Zoology (Kunming, China). Seed cells at a denseness of 1×10^4 cells/ml in Dulbecco's Modied Eagle Medium (DMEM)/F-12 medium (Gibco, Carlsbad, CA, USA) adding with 10% fetal bovine serum (FBS, Gibco), 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco). Cells were kept in a wet incubator carried 5% CO_2 and 95% air at 37 °C. Fresh medium was changed every day. Rg1 (analysis level of 97% pureness) was bought from Sigma-Aldrich (St. Louis, MO, USA), dissolved in ethanol and stored in −20 °C. Cells were pre-treated with Rg1 for 24 h, and then were treated with a series of consistence (0, 100, 200, 300, 400 and 500 μM) of H_2O_2 for 1 h [22].

2.2. CCK-8 experiment

A Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA) was used to test cell activity. Cells were seeded in 96-well plate with 5000 cells/well. After adding the CCK-8 solution, cells were kept in a wet environment with 95% air and 5% CO_2 for 1 h at 37 °C. Absorbance was tested at 450 nm via a Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.3. Apoptosis experiment

Apoptosis analysis was done through propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated Annexin V staining (BD Pharmingen, San Diego, CA, USA). Cells were cleaned in phosphate buffered saline (PBS) for three times and stained in PI/FITC-Annexin V with 50 μg/ml RNase A (Sigma-Aldrich). Cells were kept in the dark at the room temperature for 1 h. Flow cytometry analysis was made through FACS can (Beckman Coulter, Fullerton, CA, USA). Data was analyzed via FlowJo software (Tree Star Software, San Carlos, California, USA).

2.4. Transfection

MiR-216a-5p inhibitor and the relative NC were compounded by Life Technologies Corporation (Carlsbad, CA, USA) and transferred into cells. Transfection was done following the Lipofectamine 3000 reagent (Life Technologies Corporation). A 48 h period post-transfection was regarded as harvest moment in following assays.

2.5. qRT-PCR

Overall RNA was extracted through Trizol reagent (Life Technologies Corporation) and handled with DNaseI (Promega, Madison, WI, USA). Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) were used to test miR-216a-5p expression. U6 was taken as inside comparison.

2.6. Western blot

Overall protein was extracted through RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitors (Roche, Basel, Switzerland), and then quantified through BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). SDS-PAGE was used to isolate protein samples. Then, transfer the gel to the PVDF membrane. Completely immerse the membrane in a protein-free blocking solution (Sangon Biotech, Shanghai, China). Primary antibodies specific against Bax (Abcam, ab32503, Cambridge, MA, USA), pro-caspase-3 (ab183179), cleaved-caspase-3 (ab49822), pro-PARP (ab32064), cleaved-PARP (ab4830), β-actin (ab8226), beclin-1 (ab62557), p62 (ab56416), LC3-I and LC3-II (ab48394), t-phosphatidylinositol 3 kinase (PI3K) (ab140307), p-PI3K (ab182651), t-protein kinase B (AKT) (ab179463), p-AKT (ab38499), t-AMP-activated protein kinase (AMPK) (ab131512) and p-AMPK (ab23875) were cultured with the membrane for 10 min in a room kept at 4 °C overnight. The membranes were rinsed and incubated with the secondary antibodies for 1 h at room temperature. After rinsing for 6 times, add 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) to shroud film surface. The bands were gotten via Image Lab™ Software (Bio-Rad).

2.7. Statistical analysis

All assays were duplicated for 3 times. Our consequences of multiplex assays were revealed as mean ± SD. Statistical analysis was done via Graphpad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). *P*-values were counted via a one-way analysis of variance (ANOVA). *P*-value of < 0.05 indicated statistical significant data.

3. Results

3.1. Rg1 extenuated H_2O_2-induced cell activity suppression and cell apoptosis

First, we examined the concentration dependency of cell viability in the presence of the indicated H_2O_2 concentrations of 0, 100, 200, 300, 400 and 500 μM (Fig. 1A). As we expected, the cell viability was decreasing with the increasing of H_2O_2 concentration. From Fig. 1B, we found that H_2O_2 had notable inhibiting effect on cell viability when the concentration was 100 ($P < 0.05$), 200 ($P < 0.05$), 300 ($P < 0.01$), 400 ($P < 0.01$) and 500 μM ($P < 0.001$). We chose 300 μM as the working concentration in the subsequent assays because this was the cell viability semi-lethal concentration. Besides, apoptosis was notably increased by H_2O_2 ($P < 0.001$, Fig. 1C). Similarly, apoptosis relative factors (Bax, cleaved-caspase-3 and cleaved-caspase-PARP) were obviously enhanced through H_2O_2 (Fig. 1D), and standards of these factors were notably raised (all $P < 0.01$, Fig. 1E). We got that H_2O_2 could increase apoptosis.

Then we tested the function of Rg1. As shown in Fig. 1F, there was no effect on cell viability by Rg1. H_2O_2 could notably reduce cell viability ($P < 0.01$), whereas Rg1 could notably attenuate this reduction at the concentration of 200, 300 and 400 μM (all $P < 0.05$, Fig. 1G). We chose 200 μM as the working concentration in the following experiments because this was the concentration when cell viability was half restored with the great efficacy of Rg1. Besides, Rg1 attenuated apoptosis induced by H_2O_2 ($P < 0.01$, Fig. 1H) and the expression of apoptosis relative factors was weakened by Rg1 compared with H_2O_2 group (Fig. 1I). Similarly, levels of these factors were raised through H_2O_2 ($P < 0.01$, $P < 0.001$ and $P < 0.01$), whereas were decreased by Rg1 (all $P < 0.05$, Fig. 1J). So we got that Rg1 attenuated cell activity suppression and apoptosis induced by H_2O_2.

3.2. Rg1 extenuated autophagy induced by H_2O_2

For autophagy, we tested three autophagy relative factors. Beclin-1 is autophagy gene and its overexpression can stimulate autophagy [23]. Accumulation of p62 is a notable phenotype of autophagy-deficient tumor cells [24]. LC3-II is a marker for mature autophagosomes. Autophagy could be analyzed by testing the conversion of the autophagosome marker LC3-I to LC3-II [25]. Fig. 2A showed that Rg1 could weaken the H_2O_2-induced enhancement of beclin-1 and LC3-II/LC3-I. Expression of p62 was weakened by H_2O_2, while Rg1 could eliminate this mitigation (Fig. 2A). Besides, Fig. 2B revealed the notable addition of beclin-1 and LC3-II/LC3-I by H_2O_2 ($P < 0.01$ and $P < 0.001$),

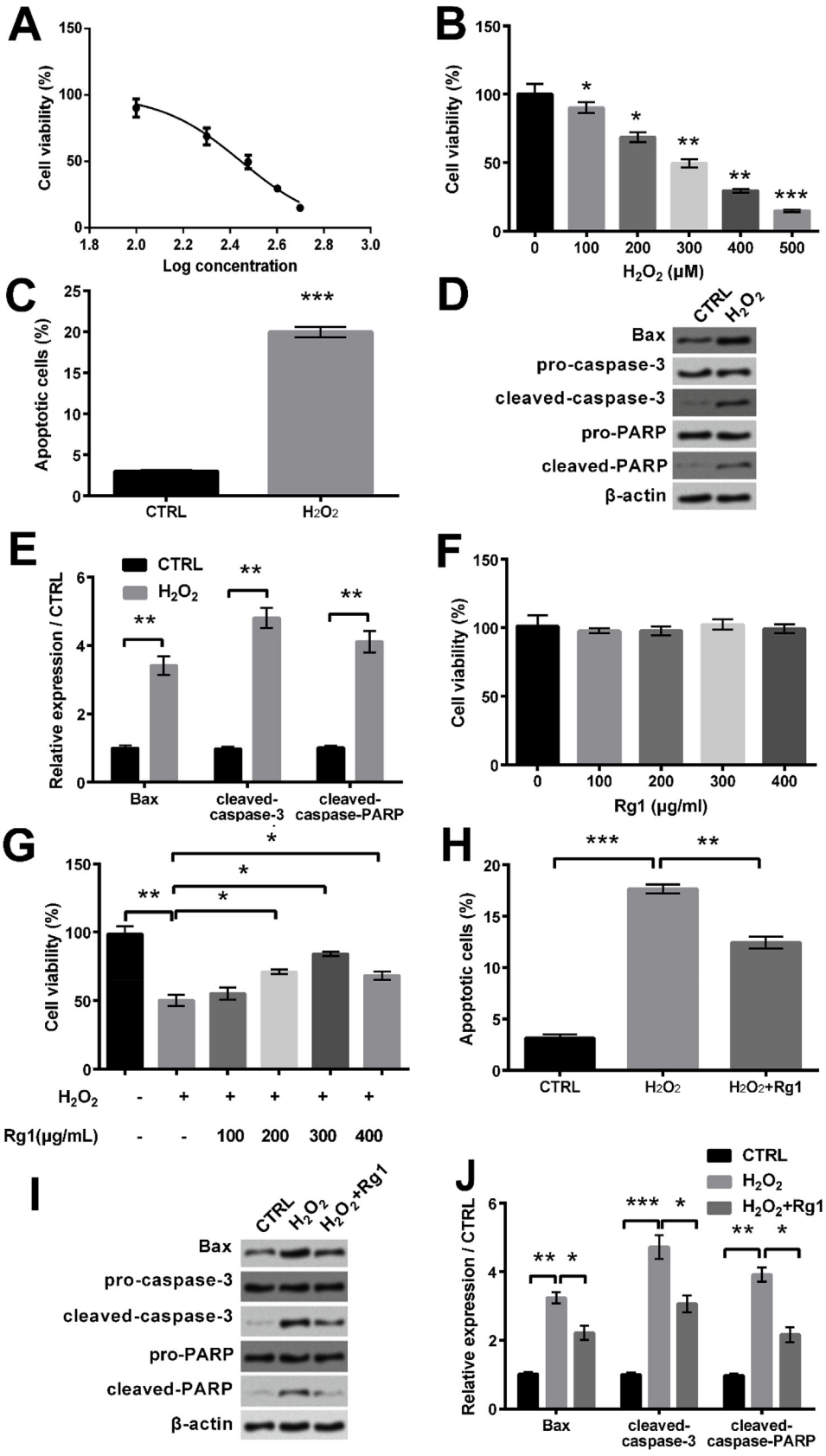

Fig. 1. Influence of Rg1 in cell activity and apoptosis caused by H_2O_2 in PC-12 cell. (A) Concentration responses timeline for the viability and H_2O_2. (B) Cell viability was tested under diverse consistence of H_2O_2 (0, 100, 200, 300, 400 and 500 μM). 300 μM was chose in the following experiments. (C) Cell apoptosis was tested via flow cytometry. (D) Expression of Bax, pro-caspase-3, cleaved-caspase-3, pro-PARP and cleaved-PARP was tested via Western blot. (E) Levels of Bax, pro-caspase-3, cleaved-caspase-3, pro-PARP and cleaved-PARP were tested via Western blot analysis. (F) Cell viability was tested via CCK-8 after treatment with Rg1. (G) Rg1 attenuated H_2O_2-induced suppression of cell viability. (H) Rg1 attenuated H_2O_2-induced cell apoptosis. (I) Expression of Bax, pro-caspase-3, cleaved-caspase-3, pro-PARP and cleaved-PARP was tested via Western blot. (J) Levels of Bax, pro-caspase-3, cleaved-caspase-3, pro-PARP and cleaved-PARP were detected via Western blot analysis. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ contrasted with control and the indicated set.

whereas those were opposite by adding of Rg1 ($P < 0.05$ and $P < 0.01$). p62 expression was notably weakened by H_2O_2 ($P < 0.05$), whereas was increased by the adding of Rg1 ($P < 0.05$, Fig. 2B). So Rg1 could attenuate H_2O_2-induced autophagy.

3.3. Rg1 positively regulated miR-216a-5p

From Fig. 3, qRT-PCR assay indicated that miR-216a-5p was notably down-regulated after H_2O_2 treatment ($P < 0.05$). But, it was

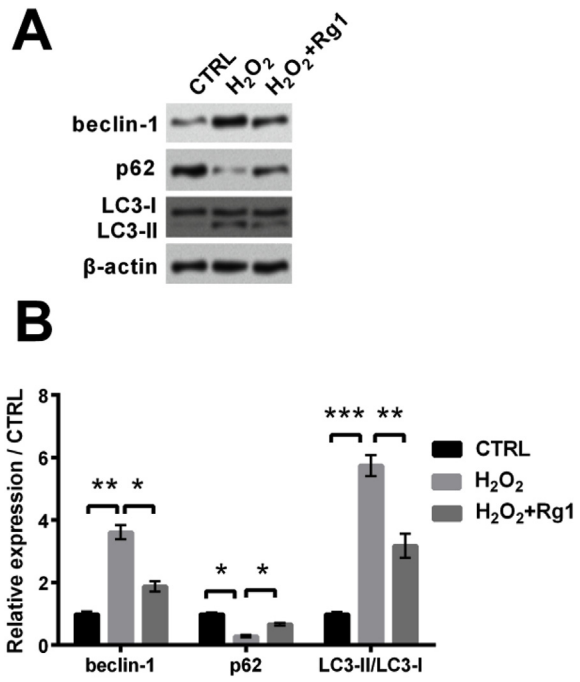

Fig. 2. Influence of Rg1 in autophagy caused by H_2O_2. (A) Standards of beclin-1, p62 and LC3-II/LC3-I were tested via Western blot. (B) Standards of beclin-1, p62 and LC3-II/LC3-I were tested via Western blot analysis. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ contrasted with indicated set.

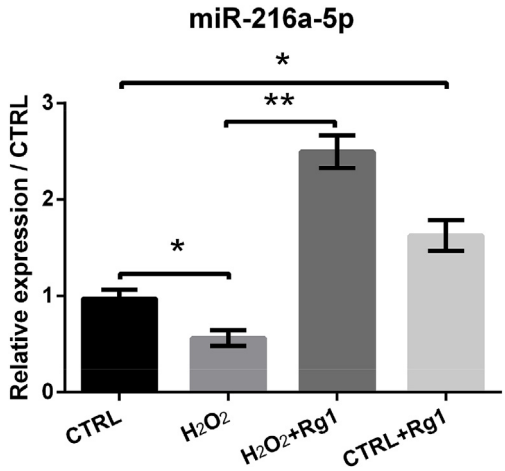

Fig. 3. Regulatory relationship between Rg1 and miR-216a-5p. RNA standard of miR-216a-5p was tested via qRT-PCR. *$P < 0.05$ and **$P < 0.01$ contrasted with indicated set.

specifically up-regulated by adding of Rg1 ($P < 0.01$).

3.4. Rg1 extenuated cell activity suppression, apoptosis and autophagy induced by H_2O_2 through up-regulating miR-216a-5p

qRT-PCR revealed that miR-216a-5p expression was notably suppressed after miR-216a-5p inhibitor transfection ($P < 0.01$, Fig. 4A). Cell viability was notably raised by Rg1 contrast with H_2O_2 set ($P < 0.05$), whereas was notably alleviated by the adding of miR-216a-5p inhibitor ($P < 0.05$, Fig. 4B). This result indicated that Rg1 attenuated H_2O_2-induced cell activity suppression by up-regulating miR-216a-5p. Besides, cell apoptosis was notably decreased by Rg1 contrast with H_2O_2 set ($P < 0.01$), whereas was notably increased by the adding of miR-216a-5p inhibitor ($P < 0.01$, Fig. 4C). Fig. 4D–E further indicated that levels of apoptosis relative factors were notably decreased through Rg1 contrast with H_2O_2 set ($P < 0.05$, $P < 0.01$ and $P < 0.01$), whereas were raised in Rg1-treated cells with miR-216a-5p inhibitor ($P < 0.05$, $P < 0.01$ and $P < 0.05$). So Rg1 reduced H_2O_2-caused apoptosis by up-regulating miR-216a-5p. Additionally, Fig. 4F–G revealed the notable attenuation of beclin-1 and LC3-II/LC3-I ($P < 0.01$ and $P < 0.001$) and the slight increase of p62 ($P < 0.05$) by Rg1 contrasted with H_2O_2 set. However, beclin-1 and LC3-II/LC3-I were notably enhanced (both $P < 0.05$) and level of p62 was notably decreased when treated with Rg1 plus miR-216a-5p inhibitor ($P < 0.05$). So we got that Rg1 attenuated H_2O_2-induced autophagy by up-regulating miR-216a-5p.

3.5. Signal pathway

Activation of PI3K/AKT is known to trigger survival signals against multiple apoptotic injury [26]. And the AMPK activation plays anti-inflammatory roles in regulating anabolism and catabolism to elevate the redox balance [27]. To further study the mechanism of Rg1, we focused on these signal pathways. Fig. 5A–B indicated that levels of p-PI3K and p-AKT were notably increased through Rg1 contrasted with H_2O_2 set ($P < 0.01$ and $P < 0.05$), whereas were notably alleviated in Rg1-treated cells with miR-216a-5p inhibitor ($P < 0.01$ and $P < 0.05$). Besides, Fig. 5C–D revealed that level of p-AMPK was notably aggravated by Rg1 compared with H_2O_2 group ($P < 0.01$), whereas was notably alleviated in Rg1-treated cells with miR-216a-5p inhibitor ($P < 0.01$). These results indicated that Rg1 elevated PI3K/AKT and AMPK pathways via positively modulating miR-216a-5p.

4. Discussion

SCI is a life-changing event. Oxidative stress is the important cause of secondary damage progression, leading to apoptosis, autophagy and loss of neurological function [28]. Therefore, defensing cells against oxidative stress injury or relieving this injury can be an effective method to cure SCI. H_2O_2 treatment was usually employed to build up cellular model of SCI [29]. For example, PC12 cells were exposed to H_2O_2 to induce cellular damage and imitate oxidative damages of SCI [20]. In our study, cell viability was notably reduced, while apoptosis and autophagy were increased after H_2O_2 treatment in PC-12 cells, indicating that H_2O_2 treatment successfully caused cell injury. Rg1, an active component of ginsenosides, has been proved to exert positive effect on anti-apoptosis [30]. But the effect of Rg1 in oxidative stress damage of SCI is unknown. We firstly researched the attenuating mechanism of Rg1 in H_2O_2-caused PC-12 cell damage. Our results suggested that Rg1 may be an effective method for the treatment of SCI.

Autophagy is an important cellular process in keeping cell homeostasis and energy production via digesting cytoplasmic components by lysosomes [31]. Previous studies have showed that autophagy is activated in SCI [32] and can produce neuroprotective effect in acute SCI through inhibition of apoptosis [33]. Here, we focused on the beneficial roles of Rg1. Rg1, the main bioactive ingredient in ginseng, has been proved to exert low toxicity [34]. Much evidence indicates that Rg1 exerts beneficial effects, such as anti-aging property [35]. As we all known, Bax may control mitochondrial permeability transition and promote the release of cytochrome c, ultimately triggering the activation of caspases, leading to apoptosis [34]. As we expected, in our study, treatment of Rg1 reduced the level of Bax, at the same time, levels of cleaved-caspase-3 and cleaved-caspase-PARP were reduced. These findings verified the anti-apoptotic function of Rg1. Moreover, Rg1 has a notable pharmacological influence in suppressing autophagy [15]. Our study were consistent with this report that Rg1 strongly inhibited autophagic factors (beclin-1 and LC3-II/LC3-I), and counteracted H_2O_2-induced p62 attenuation, leading to inhibition of H_2O_2-induced autophagy in PC-12 cells, suggesting the anti-oxidant and anti-autophagy functions of Rg1 in SCI.

To further study the mechanism of Rg1, we turn our attention to

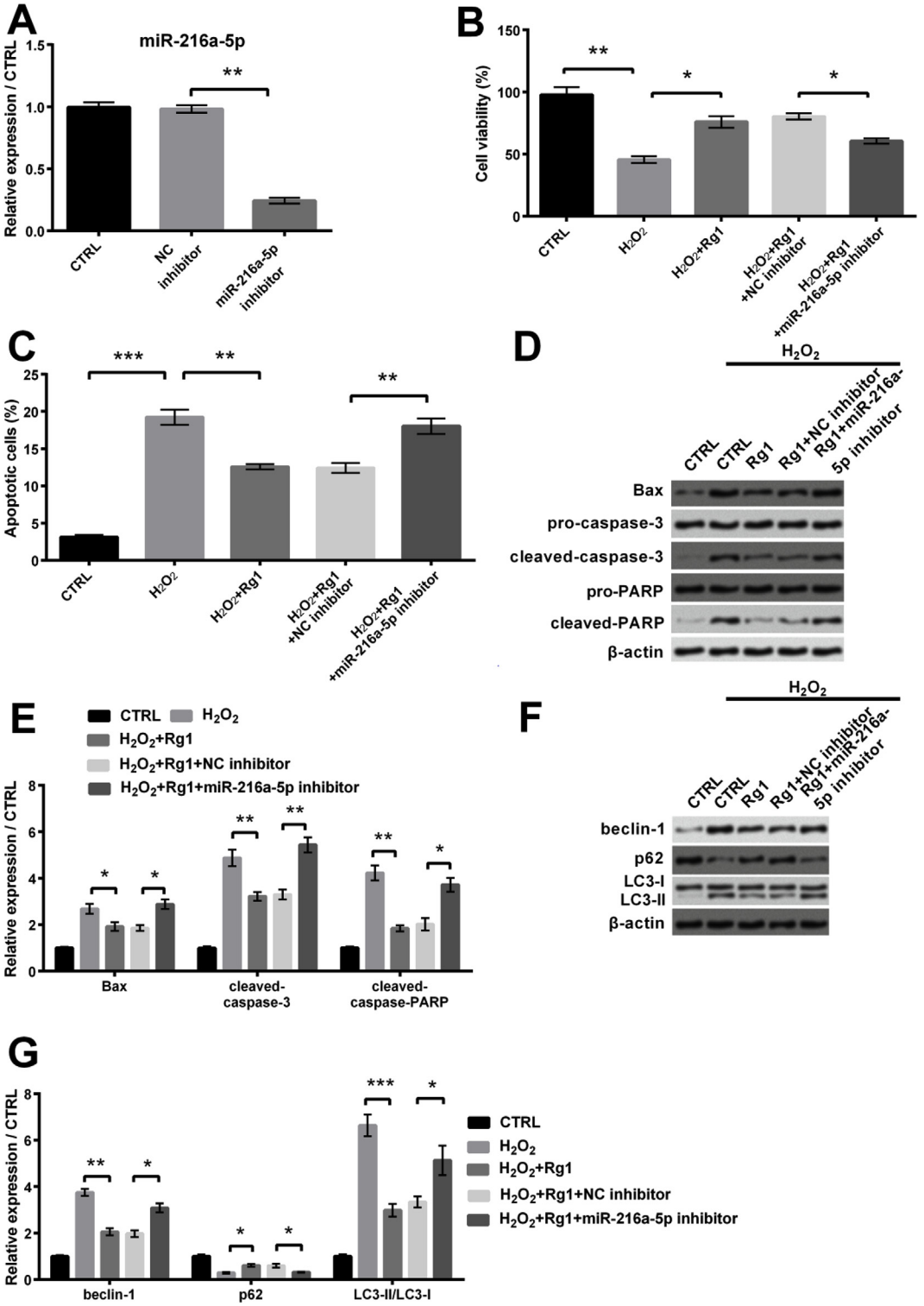

Fig. 4. Protective effects of Rg1 after transfection with Rg1 plus miR-216a-5p and relative NC in PC-12 cells. (A) RNA standard of miR-216a-5p was tested via qRT-PCR after miR-216a-5p inhibitor transfection. (B) Cell activity was tested via CCK-8. (C) Apoptosis was tested via flow cytometry. (D) Expression of Bax, pro-caspase-3, cleaved-caspase-3, pro-PARP and cleaved-PARP was tested via Western blot. (E) Levels of Bax, pro-caspase-3, cleaved-caspase-3, pro-PARP and cleaved-PARP were tested via Western blot quantitative. (F) Standards of beclin-1, p62 and LC3-II/LC3-I were tested via Western blot. (G) Standards of beclin-1, p62 and LC3-II/LC3-I were tested via Western blot analysis. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ contrasted with indicated set.

miRNA. MiRNA is important in cell growth, such as proliferation and apoptosis [36]. MiR-216a-5p, acknowledged as an oncogenic gene, is involved in tumorigenesis and development of human cancers [37]. MiR-216a-5p significantly elevated cell activity and reduced apoptosis in H_2O_2-caused 16 HBE cells of Asthma, suggesting that miR-216a-5p could regulate H_2O_2-caused damage [38]. Besides, of interest, beclin-1 was the latent mark of miR-216-5p, which could inhibit oxidized low-density lipoprotein (ox-LDL)-induced autophagy in human umbilical vein endothelial cells (HUVECs) through modulating levels of intracellular beclin-1 [39]. These reports indicate that miR-216a-5p not

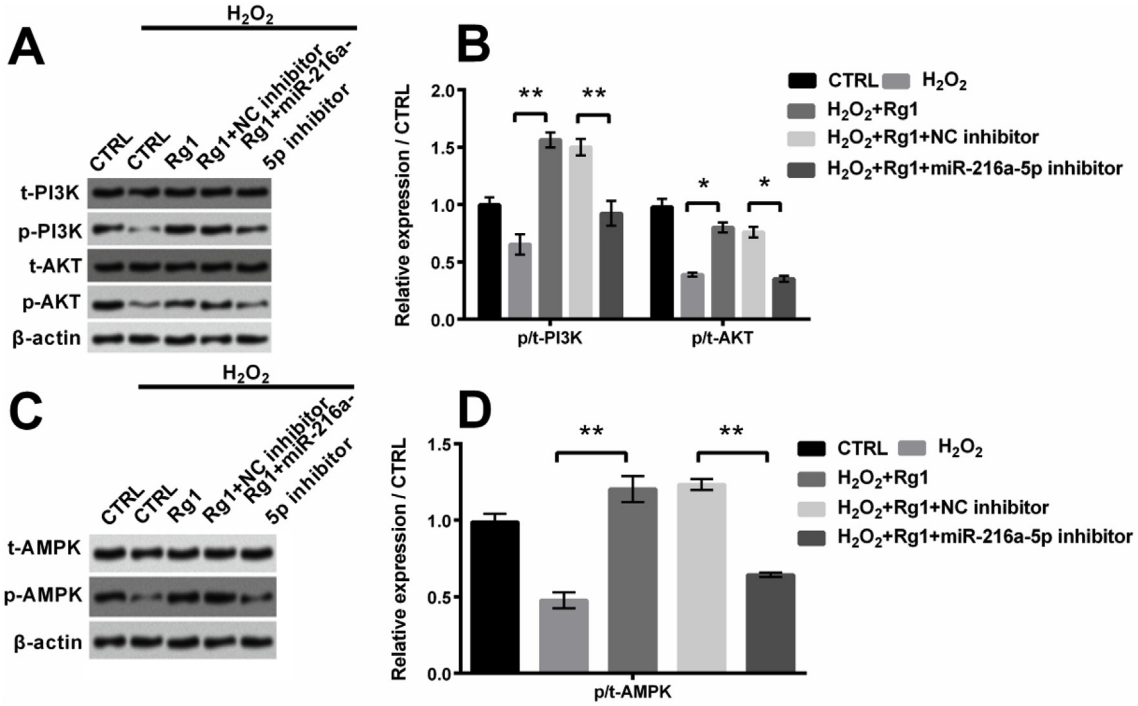

Fig. 5. Rg1 elevated PI3K/AKT and AMPK signal pathways through positively regulating miR-216a-5p after transfection with Rg1 plus miR-216a-5p and relative NC in PC-12 cells. (A) Expression of p/t-PI3K and p/t-AKT was tested via Western blot. (B) Standards of p/t-PI3K and p/t-AKT were tested via Western blot quantitative. (C) Expression of p-AMPK and t-AMPK was tested via Western blot. (D) Level of p/t-AMPK was detected via Western blot quantitative. $*P < 0.05$ and $**P < 0.01$ contrasted with indicated set.

only elevates activity, suppresses apoptosis, but also inhibits autophagy, suggesting that miR-216a-5p can reduce H_2O_2-caused cell damage. Also, functions of miR-216a-5p are similar to those of Rg1. For the first time, we found the regulation relationship between Rg1 and miR-216a-5p. Rg1 could up-regulate miR-216a-5p to attenuate cell injury induced by H_2O_2. This finding is a major discovery in SCI research.

Furthermore, the biological process is inseparable from the regulation of signal pathways. AMPK/PI3K/AKT pathways have been shown to be a key coordinator for protecting cells from oxidative and inflammatory damage [40]. PI3K is related to many cellular functions, like proliferation and apoptosis [41]. AKT is a key downstream effector of PI3K with anti-apoptotic effects [42]. AMPK is present in metabolically related organs. Cellular metabolism stimulation like cell stress can activate it [42]. Here, our findings revealed that levels of p/t-PI3K, p/t-AKT and p/t-AMPK were decreased by H_2O_2 treatment, indicating that these pathways' activations were inhibited in SCI without playing their positive roles in survival. Moreover, Lin et al. found that the formation of autophagosomes was accompanied by inhibition of the PI3K/AKT and AMPK signal pathways, indicating that there was negative regulation between these two pathways and autophagy. Importantly, our study firstly built up the relation among Rg1, miR-216a-5p and AMPK/PI3K/AKT pathways that Rg1 promoted the activation of PI3K/AKT and AMPK signal pathways through positively modulating miR-216a-5p to reduce cell damage. This regulation mechanism provides a theoretical basis for attenuating cell injury after SCI.

5. Conclusion

Our study firstly reported the underlying effects and mechanism of Rg1 on cell injury of SCI. We demonstrated that Rg1 could up-regulate miR-216a-5p, attenuate cell activity suppression, apoptosis and autophagy, and active PI3K/AKT and AMPK signal pathways to decrease H_2O_2-caused PC-12 cell damage. Because local cell injury significantly aggravates SCI, we propose that Rg1, an effective biological macromolecular, may supply a novel therapeutic approach for curing SCI.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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None.

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