Original Article

Ginsenoside Rg1 Attenuates Isoflurane-induced Caspase-3 Activation via Inhibiting Mitochondrial Dysfunction*

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Abstract

Objective The inhalation anesthetic isoflurane has been shown to induce mitochondrial dysfunction and caspase activation, which may lead to learning and memory impairment. Ginsenoside Rg1 is reported to be neuroprotective. We therefore set out to determine whether ginsenoside Rg1 can attenuate isoflurane-induced caspase activation via inhibiting mitochondrial dysfunction.

Methods We investigated the effects of ginsenoside Rg1 at concentrations of 12.5, 25, and 50 μmol/L and pretreatment times of 12 h and 24 h on isoflurane-induced caspase-3 activation in H4 naïve and stably transfected H4 human neuroglioma cells that express full-length human amyloid precursor protein (APP) (H4-APP cells). For mitochondrial dysfunction, we assessed mitochondrial permeability transition pore (mPTP) and adenosine-5'-triphosphate (ATP) levels. We employed Western blot analysis, chemiluminescence, and flowcytometry.

Results Here we show that pretreatment with 50 μmol/L ginsenoside Rg1 for 12 h attenuated isoflurane-induced caspase-3 activation and mitochondrial dysfunction in H4-APP cells, while pretreatment with 25 and 50 μmol/L ginsenoside Rg1 for 24 h attenuated isoflurane-induced caspase-3 activation and mitochondrial dysfunction in both H4 naïve and H4-APP cells.

Conclusion These data suggest that ginsenoside Rg1 may ameliorate isoflurane-induced caspase-3 activation by inhibiting mitochondrial dysfunction. Pending further studies, these findings might recommend the use of ginsenoside Rg1 in preventing and treating isoflurane-induced neurotoxicity.

Key words: Ginsenoside Rg1; Isoflurane; Neurotoxicity; Mitochondrial dysfunction

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INTRODUCTION

After surgery and anesthesia, some patients show a decline in cognitive function. This condition is called postoperative cognitive dysfunction (POCD), and is an impairment of memory, concentration, language comprehension, and social integration[1]. A distressing complication after surgery, POCD is independently associated with poor short-term and long-term outcomes. Anesthesia and surgery have been reported to induce cognitive dysfunction, to which patients with Alzheimer's disease (AD) or older patients are susceptible[2], but it can develop in all age groups. However, there is still a lack of effective treatments for POCD, and many studies aim to find new and novel drugs to treat or prevent POCD[3].

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The exact pathogenesis of POCD is complex and multifactorial; however, it appears to share certain pathological markers with AD including amyloid β deposition, caspase activation, and cell apoptosis[4-5]. Animal models have shown that anesthetics, particularly inhalational anesthetics, can increase the development of these pathological markers in the brain[4]. Isoflurane, a common inhalational anesthetic, could induce caspase activation and apoptosis through the mitochondrial dependent apoptosis pathway[6], and current studies suggest that caspase activation (without apoptosis) can induce microglia activation, contributing to neuropathogenesis of AD[7]. Mitochondrial dysfunction can lead to caspase activation and apoptosis, potentially through the opening of mPTP, reductions in mitochondrial membrane potential, and decreases in ATP concentration, leading to cytotoxicity and impairment of learning and memory[8-10].

Ginseng root has been used for several thousand years as a highly valued herb to treat weakness and fatigue, especially in China. The major active components of ginseng are ginsenosides, a diverse group of steroidal saponins, which target myriad tissues, producing an array of pharmacological responses[11]. Ginsenosides include Rb1, Rb2, Rc, Rd, Re, Rg1, and Rg2, with Rg1 being one of the most studied components. Ginsenoside Rg1 exerts a neuroprotective effect and is beneficial in AD models *in vivo* and *in vitro*[12-13]. Ginsenoside Rg1, used as a small-molecule drug, can improve learning and memory in animals[14-15], inhibit apoptosis induced by amyloid β[16], alleviate oxidative stress[17], inhibit β-secretase activity[13], maintain neuron activity at a normal level in the hippocampus of amouse model of amyloid-β-induced dementia[18], and improve neuralplasticity[19]. Moreover, ginsenoside Rg1 has recently been used to attenuate oligomeric amyloid β_{1-42}-induced mitochondrial dysfunction[20]. Ginsenoside Rg1 may be a candidate neuroprotective agent for treating AD or aging and memory deterioration[19]. Having few side effects, it can also be used for prevention or treatment for all age groups. However, whether ginsenoside Rg1 has any protective effect on isoflurane-induced neurotoxicity and mitochondrial dysfunction has not yet been reported. In this study, we assessed the effects of different doses and pretreatment times of ginsenoside Rg1 on isoflurane-induced caspase-3 activation in H4 naïve and H4-APP cells. In addition, we performed mechanistic studies to determine whether ginsenoside Rg1 can attenuate isoflurane-induced caspase-3 activation via inhibiting mPTP opening and increasing ATP concentration.

MATERIALS AND METHODS

Cells

We employed H4 naïve and H4-APP cells. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 9% heat-inactivated fetal calfserum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L-glutamine; H4-APP cells were supplemented with 220 μg/mL G418.

Treatments for H4 Naïve and H4-APP Cells

Isoflurane was delivered from an anesthesia machine to a sealed plastic box in a 37 °C incubator containing six-well plates seeded with one million cells in 1.5 mL cell culture media. A compact anesthesia monitor (Datex-Ohmeda, GE Healthcare, Finland) was used to continuously monitor the delivered concentrations of CO_2, O_2, and isoflurane. Cells were treated with 2% isoflurane plus 21% O_2 and 5% CO_2 for 6 h, as described by Zhang et al.[21], for the purpose of measuring caspase-3 activation. The cultured cells were treated for 3 h in the studies, to measure mPTP opening and ATP levels without cell death, as described by Zhang et al.[22]. Ginsenoside Rg1 was obtained from the National Institutes for Food and Drug Control (Beijing, China) in the form of white powder-like crystals, and has a molecular weight of 800 and general formula $C_{42}H_{72}O_{14}$. In the interaction experiments, ginsenoside Rg1 was dissolved in DMEM without serum at 4 °C. Cells were pretreated with 12.5, 25, or 50 μmol/L ginsenoside Rg1 for 12 h or 24 h or DMEM as control before isoflurane treatment, as well as during isoflurane treatment.

Cell Lysis and Protein Amount Quantification

Cell pellets were detergent-extracted on ice using an immunoprecipitation buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% Nonidet P-40) plus protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A). The lysates were collected, and centrifuged at 13,000 rpm for 15 min. Total protein content was determined using a bicinchoninic acid protein assay kit (Thermo scientific, Rockford, USA).

Western Blot Analysis

The harvested H4 naïve and H4-APP cells were subjected to Western blot analyses as described by Zhang et al.[22]. A caspase-3 antibody (1:1000 dilution; Cell Signaling Technology, Inc., Danvers, MA) was used to recognize full-length (FL) caspase-3 (35-40 kDa) and caspase-3 fragment (17-20 kDa) resulting from cleavage at aspartate position 175. Antibody anti-β-actin(1:10,000, Sigma, St. Louis, MO) was used to detect β-actin (42 kDa). Results were obtained from three independent experiments. The intensity of the signals was analyzed using the Quantity One program (version 4.62). Caspase-3 normalization was performed by determining the ratio of caspase-3 fragment to FL caspase-3, and changes in levels of caspase-3 in treated cells were presented as percentages of the corresponding levels in control cells.

ATP Measurement

We employed an ATP determination kit (Invitrogen, Carlsbad, CA) in experiments to detect ATP levels according to a protocol provided by the company. Briefly, cells were incubated in six-well plates for the desired pretreated times with ginsenoside Rg1. The cells were then exposed to isoflurane treatment. At the end of the treatment, the amount of fluorescence was measured and the levels of ATP in the experimental samples were calculated by comparison with a standard curve, made from samples containing known concentrations of ATP.

Flowcytometric Analysis of mPTP Opening

Cells were treated with 2% isoflurane for 3 h. The opening of mPTP was determined by flowcytometry, using a MitoProbe™ transition pore assay kit (Invitrogen, Carlsbad, CA). In normal conditions, non-fluorescent acetoxymethyl ester (AM) of calcein dye (calcein AM) and cobalt can enter the cell. The AM groups are cleaved from calcein via non-specific esterase, and calcein then fluoresces in both the cytosol and mitochondria. Cobalt can quench the cytosolic calcein fluorescence. However, cobalt cannot enter healthy mitochondria freely, and therefore cannot quench the mitochondrial calcein signal. When the mPTP open, cobalt enters through it and subsequently quenches the mitochondrial calcein signal. Flowcytometry was used to detect the amount of cells that exhibit quenched calcein signals inside the mitochondria. The position of the curves indicates the amount of such cells, and thus indicates the opening of mPTP. Ionomycin was used as a positive control for the opening of mPTP. Dead cells and debris were excluded from analysis by setting gates on forward and side angle light scatter.

Statistical Analysis

All numerical results were expressed as mean±standard deviation. Data were analyzed by analysis of variance (ANOVA) followed by analysis of significance (least significant difference test) for multiple time points groups. Probability values <0.05 were considered statistically significant. Statistical analysis was carried out using SPSS (version 17.0 for Windows).

RESULTS

Effect of Ginsenoside Rg1 with 12 h Pretreatment on Isoflurane-induced Caspase-3 Activation in H4-APP and H4 Naïve Cells

The H4-APP cells were treated with 12.5, 25, 50 μmol/L ginsenoside Rg1 or DMEM for 12 h followed by 2% isoflurane or control condition for 6 h. The cells were harvested at the end of the experiment and were subjected to Western blot analysis. Quantification of the Western blot (Figure 1B) revealed that isoflurane (Bar 5) led to caspase-3 activation, as compared with the control condition (Bar 1) in H4-APP cells: 7.23 *vs.* 1.00 fold ($^{*}P$=0.000). Treatment with 50 μmol/L Rg1 (Bar 8) attenuated isoflurane-induced caspase-3 activation (Bar 5): 4.02 *vs.* 7.23 fold ($^{\#}P$=0.029). These findings suggest that pretreatment with 50 μmol/L ginsenoside Rg1 for 12 h might mitigate isoflurane-induced caspase-3 activation in H4-APP cells.

Next, we assessed whether ginsenoside Rg1 pretreated for 12 h could also attenuate isoflurane-induced caspase-3 activation in H4 naïve cells, which have lower amyloid β levels than H4-APP cells. Quantification of the Western blot (Figure 1D) showed that isoflurane (Bar 5) led to caspase-3 activation, as compared with the control condition (Bar 1) in H4 naïve cells: 4.75 *vs.* 1.00 fold ($^{*}P$=0.000). Treatment with 12.5, 25, and 50 μmol/L Rg1 (Bars 6, 7, and 8) did not attenuate isoflurane-induced caspase-3 activation (Bar 5) in H4 naïve cells: 4.47, 4.29, and 4.21, respectively, *vs.* 4.75 fold (P=0.60, 0.45, and 0.31, respectively). These results showed that ginsenoside Rg1 did not mitigate

isoflurane-induced caspase-3 activation in H4 naïve cells. Taken together, these findings suggest that pretreatment with 50 μmol/L ginsenoside Rg1 for 12 h might have different effects on isoflurane-induced caspase-3 activation in H4-APP and H4 naïve cells.

Effect of Ginsenoside Rg1 with 24 h Pretreatment on Isoflurane-induced Caspase-3 Activation in H4-APP and H4 Naïve Cells

We performed relevance studies by pretreatment with 12.5, 25, and 50 μmol/L ginsenoside Rg1 for 24 h in H4-APP cells. Quantification of the Western blot (Figure 2B) showed that isoflurane (Bar 5) led to caspase-3 activation, as compared with the control condition (Bar 1) in H4-APP cells: 8.16 *vs.* 1.00 fold ($^{*}P$=0.001). Treatment with 12.5 μmol/L Rg1 (Bar 6) did not attenuate isoflurane-induced caspase-3 activation (Bar 5): 5.21 *vs*. 8.16 fold (P=0.10), but treatment with 25, and 50 μmol/L Rg1 (Bars 7 and 8) attenuated isoflurane-induced caspase-3 activation: 4.30 and 2.99, respectively, *vs.* 8.16 fold ($^{\#}P$= 0.036, 0.007, respectively). These findings suggest that

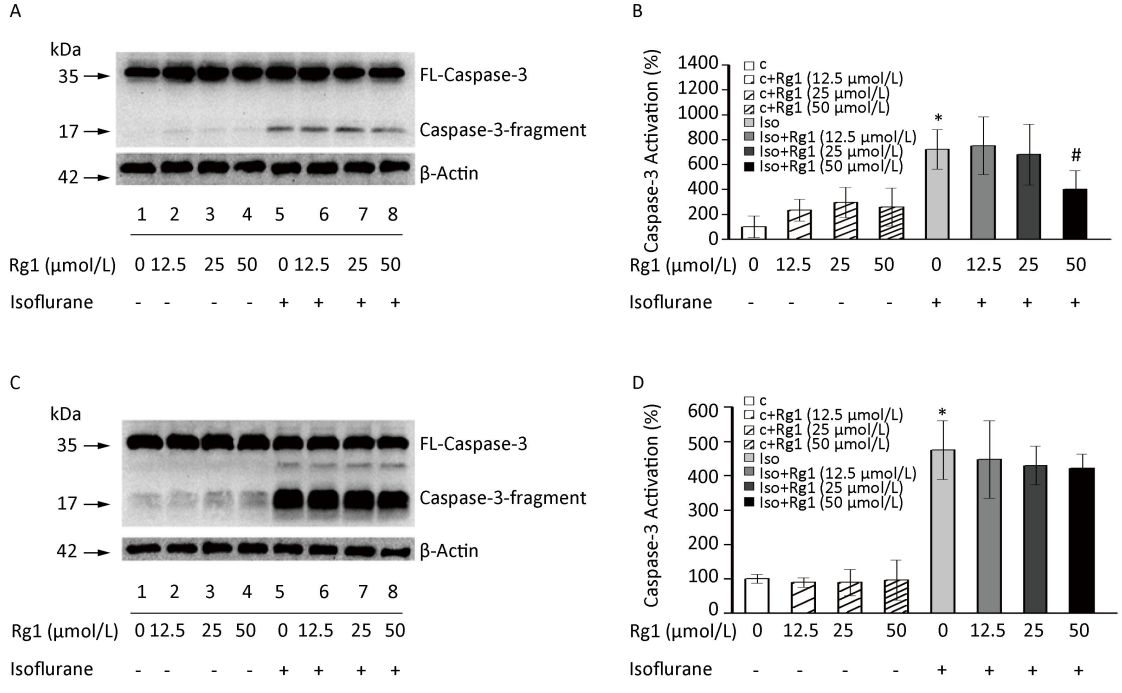

Figure 1. Effect of ginsenoside Rg1 with 12 h pretreatment on isoflurane-induced caspase-3 activation in H4-APP and H4 naïve cells. (A) Western blotting shows that treatment with 2% isoflurane for 6 h (Lane 5) induces caspase-3 activation as compared with the control condition (Lane 1) in H4-APP cells. 50 μmol/L Rg1 treatment attenuates isoflurane-induced caspase-3 activation (Lane 8) as compared with isoflurane treatment (Lane 5) in H4-APP cells. (B) Quantification of the Western blot shows that isoflurane (Bar 5) induces caspase-3 activation, as compared with the control condition (Bar 1) in H4-APP cells. Treatment with 50 μmol/L Rg1 (Bar 8) attenuates isoflurane-induced caspase-3 activation, as compared with isoflurane treatment (Bar 5) in H4-APP cells. (C) Western blotting shows that treatment with 2% isoflurane for 6 h (Lane 5) induces caspase-3 activation, as compared with the control condition (Lane 1) in H4 naïve cells, but that treatment with 12.5, 25, or 50 μmol/L Rg1 does not attenuate isoflurane-induced caspase-3 activation (Lanes 6, 7, and 8), as compared with isoflurane treatment (Lane 5) in H4 naïve cells. (D) Quantification of the Western blot shows that isoflurane (Bar 5) induces caspase-3 activation, as compared with the control condition (Bar 1) in H4 naïve cells, but that treatment with 12.5, 25, and 50 μmol/L Rg1 (Bars 6, 7, and 8) does not attenuate isoflurane-induced caspase-3 activation, as compared with isoflurane treatment (Bar 5) in H4 naïve cells. **vs.* control group, P<0.05, $^{\#}$*vs.* isoflurane treatment group, P<0.05.

pretreatment with 25 and 50 μmol/L ginsenoside Rg1 for 24 h might mitigate isoflurane-induced caspase-3 activation in H4-APP cells.

Next, we performed the same treatment in H4 naïve cells. Quantification of the Western blot (Figure 2D) showed that isoflurane (Bar 5) led to caspase-3 activation, as compared with the control condition (Bar 1) in H4 naïve cells: 4.57 *vs.* 1.00 fold ($^{*}P$=0.000). Treatment with 12.5 μmol/L Rg1 (Bar 6) did not attenuate isoflurane-induced caspase-3 activation (Bar 5): 4.09 *vs.* 4.57 fold (P=0.53), but treatment with 25 and 50 μmol/L Rg1 (Bars 7 and 8) attenuated isoflurane-induced caspase-3 activation (Bar 5): 2.95 and 2.70, respectively, versus 4.57 fold ($^{\#}P$=0.044 and 0.024, respectively). Taken together, these findings suggest that pretreatment with 25 and 50 μmol/L ginsenoside Rg1 for 24 h might have similar effects in affecting isoflurane-induced caspase-3 activation between the H4-APP and H4 naïve cells.

Effect of Ginsenoside Rg1 with 12 h Pretreatment on Isoflurane-induced Mitochondrial Dysfunction in H4-APP and H4 Naïve Cells

Ginsenoside Rg1 can attenuate isoflurane-induced caspase-3 activation, and isoflurane-induced caspase-3 activation may result from isoflurane-induced mitochondrial dysfunction. Next, we asked whether ginsenoside Rg1 can attenuate isoflurane-induced caspase-3 activation through the mitochondrial pathway. To determine the effect of Rg1 on mitochondrial function and properties, we measured the ATP level and mPTP opening.

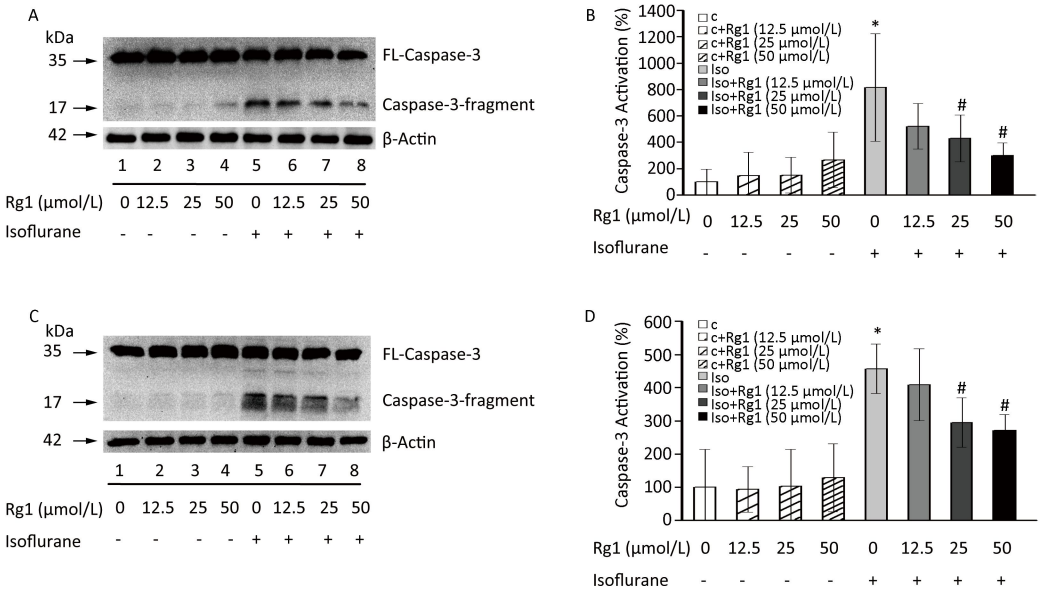

Figure 2. Effect of ginsenoside Rg1 with 24 h pretreatment on isoflurane-induced caspase-3 activation in H4-APP and H4 naïve cells. (A) Western blotting shows that treatment with 2% isoflurane for 6 h (Lane 5) induces caspase-3 activation, as compared with the control condition (Lane 1) in H4-APP cells. Treatment with 25 and 50 μmol/L Rg1 attenuates isoflurane-induced caspase-3 activation (Lanes 7 and 8), as compared with isoflurane treatment (Lane 5) in H4-APP cells. (B) Quantification of the Western blot shows that treatment with 25 and 50 μmol/L Rg1 (Bars 7 and 8) attenuates the isoflurane-induced caspase-3 activation (Bar 5). (C) Western blotting shows that treatment with 2% isoflurane for 6 h (Lane 5) induces caspase-3 activation, as compared with the control condition (Lane 1) in H4 naïve cells. Treatment with 25 and 50 μmol/L Rg1 attenuates isoflurane-induced caspase-3 activation (Lanes 7 and 8), as compared with isoflurane treatment (Lane 5) in H4 naïve cells. (D) Quantification of the Western blot shows that treatment with 25 and 50 μmol/L Rg1 (Bars 7 and 8) attenuates isoflurane-induced caspase-3 activation, as compared with isoflurane treatment. **vs.* control group, P<0.05, $^{\#}$*vs.* isoflurane treatment group, P<0.05.

The H4-APP cells were treated with 12.5, 25, and 50 μmol/L ginsenoside Rg1 or DMEM for 12 h followed by 2% isoflurane or control conditions for 3 h, which would decrease ATP levels without cell death[22]. As shown in Figure 3A, ATP generation was decreased for cells treated with isoflurane (Bar 5), as compared with the control group (Bar 1): 0.41 *vs.* 1.00 fold ($^{*}P$=0.000). However, after pretreatment with Rg1 for 12 h, the ATP levels were increased compared with the isoflurane-treated group, especially for cells pretreated with 50 μmol/L Rg1 (Bar 8): 0.68 *vs.* 0.41 fold ($^{\#}P$=0.019). Second, we measured mPTP opening. Flow cytometry of calcein AM and cobalt showed that treatment with 50 μmol/L Rg1 (Figure 4A, Peak 5) led to reductions in isoflurane-induced mPTP opening (Figure 4A, Peak 2), as evidenced by the right shift of the curve, whereas treatment with 12.5 and 25 μmol/L Rg1 (Figure 4A, Peaks 3 and 4) did not affect the opening of mPTP. Quantification of the fluorescence intensity (Figure 4B) showed that 50 μmol/L Rg1 (Bar 5) led to reductions in isoflurane-induced mPTP opening: 3.25 *vs.* 1.39 ($^{\#}P$=0.000). The change of mPTP opening correlated with the change of ATP level in H4-APP cells. Overall, the change of mitochondrial function correlated with the change of caspase-3 activation for 50 μmol/L ginsenoside Rg1 pretreatment in H4-APP cells.

The H4 naïve cells were treated in the same way. As shown in Figure 3B, ATP generation treated with isoflurane (Bar 5) decreased, as compared with the control group (Bar 1): 0.43 *vs.* 1.00 fold ($^{*}P$=0.002) in H4 naïve cells. However, pretreatment with 12.5, 25, and 50 μmol/L Rg1 (Bars 6, 7, and 8) for 12 h did not affect the ATP levels, as compared with the isoflurane-treated group (Bar 5): 0.52, 0.58, and 0.65, respectively, versus 0.43 fold (P=0.56, 0.35, and 0.17, respectively). Flow cytometry of calcein AM and cobalt showed that the treatment with 12.5 and 25 μmol/L Rg1 (Figure 4C, Peaks 3 and 4) did not lead to reductions in the isoflurane-induced mPTP opening (Figure 4C, Peak 2), whereas pretreatment with 50 μmol/L Rg1 (Figure 4C, Peak 5) led to a right shift of the curve. Quantification of the fluorescence intensity (Figure 4D) showed that 50 μmol/L Rg1 (Bar 5) led to reductions in isoflurane-induced mPTP opening (Bar 2): 3.13 *vs.* 1.23 ($^{\#}P$=0.000) in H4 naïve cells. The change of ATP level correlated with the change of caspase-3 activation, but the change of mPTP opening was different from the changes of ATP level and caspase-3 activation for 50 μmol/L ginsenoside Rg1 pretreatment in H4 naive cells.

Effect of Ginsenoside Rg1 with 24 h Pretreatment on Isoflurane-induced Mitochondrial Dysfunction in H4-APP and H4 Naïve Cells

We performed relevance studies by pretreatment with 12.5, 25, and 50 μmol/L ginsenoside Rg1 for 24 h in H4-APP cells. As can be seen in Figure 5A, ATP generation treated with isoflurane (Bar 5) decreased, as compared with the control group (Bar 1): 0.13 *vs.* 1.00 fold ($^{*}P$=0.001). When cells were pretreated with 25 and

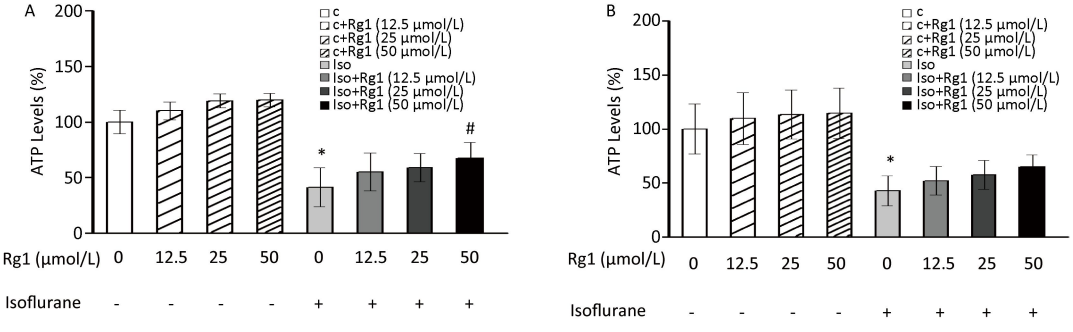

Figure 3. Effect of 12 h pretreatment with ginsenoside Rg1 on ATP levels in H4-APP and H4 naïve cells. (A) Isoflurane (Bar 5) reduces ATP level, as compared with the control condition (Bar 1) in H4-APP cells. Pretreatment with 50 μmol/L Rg1 for 12h (Bar 8) increases the ATP level, as compared with the isoflurane-treated group (Bar 5) in H4-APP cells. (B) Isoflurane (Bar 5) reduces ATP level as compared with the control condition (Bar 1) in H4 naïve cells. Pretreatment with 12.5, 25, and 50 μmol/L Rg1 for 12 h (Bars 6, 7, and 8) does not increase ATP levels, as compared with the isoflurane-treated group (Bar 5) in H4 naïve cells. **vs.* control group, P<0.05, $^{\#}$*vs.* isoflurane treatment group, P<0.05.

50 μmol/L Rg1 for 24 h, ATP levels (Bars 7 and 8) increased, as compared with the isoflurane-treated group (Bar 5): 0.72 and 0.89, respectively, versus 0.13 fold ($^{\#}P$=0.01 and 0.002, respectively). Flow cytometry of calcein AM and cobalt (Figure 6A) showed that treatment with 25 and 50 μmol/L Rg1 (Figure 6A, Peaks 4 and 5) led to a reduction in isoflurane-induced mPTP opening (Figure 6A, Peak 2). Quantification of fluorescence intensity (Figure 6B) showed that 25 and 50 μmol/L Rg1 (Bars 4 and 5) led to reductions in isoflurane-induced mPTP opening (Bar 2): 4.88 and 6.49 versus 1.37 ($^{\#}P$=0.002 and 0.000, respectively).

For H4 naïve cells (Figure 5B), ATP generation decreased after treatment with isoflurane (Bar 5), as compared with the control group (Bar 1): 0.25 *vs.* 1.00 fold ($^{*}P$=0.000). After pretreatment with 25 and 50 μmol/L Rg1 for 24 h, ATP levels (Bars 7 and 8) were

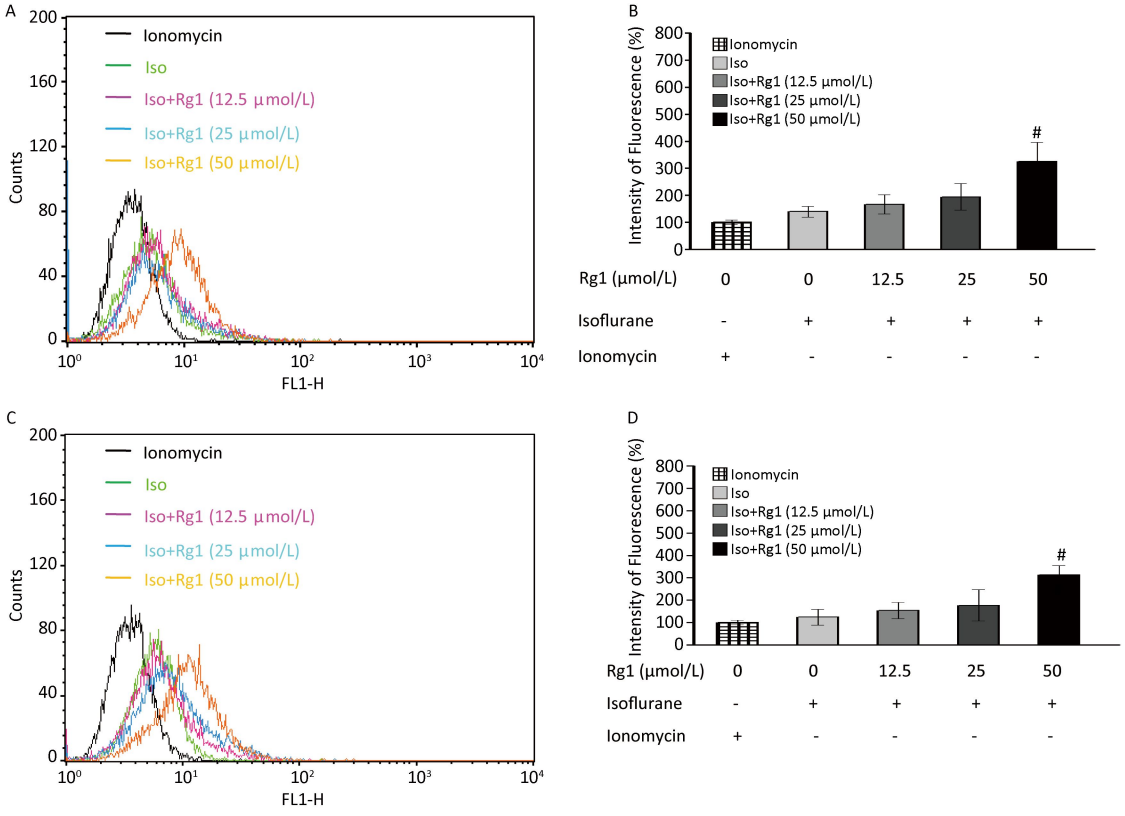

Figure 4. Effect of ginsenoside Rg1 with 12 h pretreatment on isoflurane-induced mPTP opening in H4-APP and H4 naïve cells. (A) Flow cytometry shows changes in calcein levels in mitochondria of H4-APP cells stained with calcein AM plus cobalt, indicating the opening of mPTP. Peak 1: treatment with ionomycin (positive control of mPTP opening); Peak 2: treatment with isoflurane; Peak 3: treatment with isoflurane plus 12.5 μmol/L Rg1; Peak 4: treatment with isoflurane plus 25 μmol/L Rg1; Peak 5: treatment with isoflurane plus 50 μmol/L Rg1. Treatment with 50 μmol/L Rg1 attenuates isoflurane-induced opening of mPTP in H4-APP cells, as demonstrated by the right shift of the curve. (B) Quantification of fluorescence intensity shows that 50 μmol/L Rg1 (Bar 5) leads to a reduction in isoflurane-induced mPTP opening (Bar 2) in H4-APP cells. (C) Flow cytometry shows changes in calcein levels in mitochondria of H4 naïve cells. Peak 1: treatment with ionomycin; Peak 2: treatment with isoflurane; Peak 3: treatment with isoflurane plus 12.5 μmol/L Rg1; Peak 4: treatment with isoflurane plus 25 μmol/L Rg1; Peak 5: treatment with isoflurane plus 50 μmol/L Rg1. Treatment with 50 μmol/L Rg1 attenuates isoflurane-induced opening of mPTP in H4 naïve cells, as demonstrated by the right shift of the curve. (D) Quantification of fluorescence intensity shows that 50 μmol/L Rg1 (Bar 5) leads to a reduction in isoflurane-induced mPTP opening (Bar 2) in H4 naïve cells. $^{\#}$*vs.* isoflurane treatment group, P<0.05.

increased, compared with that of the isoflurane-treated group (Bar 5) in H4 naïve cells: 0.62 and 0.82, respectively, versus 0.25 fold ($^{\#}P$=0.021 and 0.001, respectively). Flow cytometry of calcein AM and cobalt (Figure 6C) showed that treatment with 25 and 50 μmol/L Rg1 (Figure 6C, Peaks 4 and 5) led to reductions in isoflurane-induced mPTP opening (Figure 6C, Peak 2) in H4 naïve cells. Quantification of the fluorescence intensity (Figure 6D) showed that 25 and 50 μmol/L Rg1 (Bars 4 and 5) led to reductions in isoflurane-induced mPTP opening(Bar 2) in H4 naïve cells: 4.51 and 5.98, respectively, versus 1.37 ($^{\#}P$=0.000 and 0.000, respectively). Taken together, these findings suggest that pretreatment with 25 and 50 μmol/L ginsenoside Rg1 for 24 h might have the same effects on ATP level and mPTP opening for H4-APP and H4 naïve cells. The changes of mitochondrial function also correlated with the changes of caspase-3 activation in H4-APP and H4 naïve cells.

DISCUSSION

Current studies have shown that the common inhalation anesthetic isoflurane might induce caspase-3 activation and neurotoxicity *in vitro*[23] and *in vivo*[24], which may lead to learning and memory impairment in mice[25] and cognitive dysfunction in human beings[26]. Our results are consistent with the published observation that isoflurane results in caspase-3 activation. Mitochondria function as 'cellular power plants' because they generate most of the cellular ATP, which is used as a source of chemical energy. Specifically, isoflurane may induce caspase-3 activation through the mitochondrial pathway. Zhang et al.[22] suggest that isoflurane-induced opening of mPTP could be among the underlying mechanisms by which isoflurane induces caspase-3 activation and neurotoxicity. Cyclosporin A, a blocker of mPTP opening[27-29], can attenuate isoflurane-induced mitochondrial dysfunction and caspase-3 activation. However, in the search for a strategy to prevent and treat isoflurane neurotoxicity, cyclosporin A is not routinely used in patients, owing to its nephrotoxicity, hepatotoxicity, and cardiotoxicity side effects[30]. Therefore, it is important to assess whether other mPTP inhibitors can also attenuate isoflurane-induced neurotoxicity.

As one of the main active ingredients in ginseng, Rg1 is generally regarded to be beneficial for neurodegenerative diseases, with few side effects. Previous studies have demonstrated that ginsenoside Rg1 has effective anti-amnesic, anti-aging, and anti-oxidant effects[19,31], but it is unclear whether Rg1 has a protective effect on isoflurane-induced mitochondrial dysfunction. We have found that ginsenoside Rg1 can attenuate isoflurane-induced caspase-3 activation *in vitro*. These results suggest that ginsenoside Rg1 might attenuate isoflurane-induced neurotoxicity. In our mechanistic studies, we have shown that ginsenoside Rg1 can inhibit isoflurane-induced decreases in ATP concentration and mPTP opening. Zhang et al.[22] have revealed that isoflurane might induce caspase activation, apoptosis, and learning and memory impairment by inducing mitochondrial dysfunction (e.g., mPTP opening and decrease in ATP

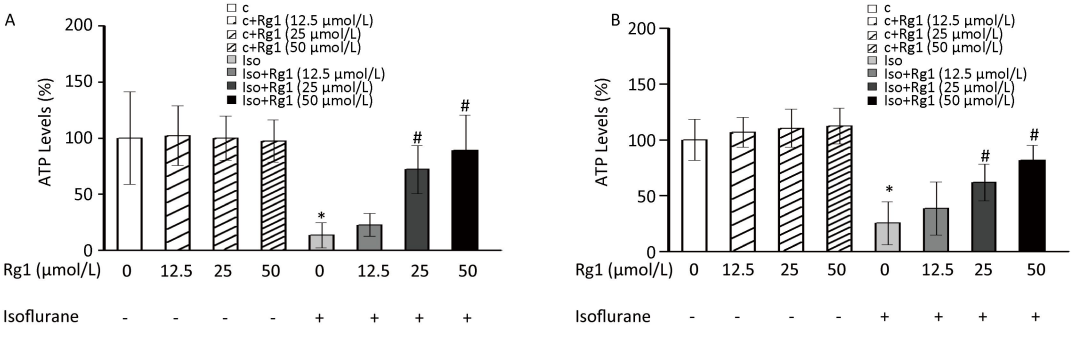

Figure 5. Effect of pretreatment with ginsenoside Rg1 for 24 h on ATP levels in H4-APP and H4 naïve cells. (A) Isoflurane (Bar 5) reduces ATP level, as compared with the control condition (Bar 1) in H4-APP cells. Pretreatment with 25 and 50 μmol/L Rg1 for 24 h (Bars 7 and 8) increases ATP levels, as compared with the isoflurane-treated group (Bar 5) in H4-APP cells. (B) Isoflurane (Bar 5) reduces ATP level, as compared with the control condition (Bar 1) in H4 naïve cells. Pretreatment with 25 and 50 μmol/L Rg1 for 24 h (Bars 7 and 8) increases ATP levels, as compared with the isoflurane-treated group (Bar 5) in H4 naïve cells. **vs.* control group, P<0.05, $^{\#}$*vs.* isoflurane treatment group, P<0.05.

concentrations). Collectively, these findings suggest that ginsenoside Rg1 might mitigate isoflurane-induced caspase-3 activation by inhibiting mitochondrial dysfunction, although further studies are required. Note that treatment with 2% isoflurane for a short duration (e.g., 3 h) induces opening of mPTP and reduction of ATP without caspase-3 activation and cell death, whereas treatment with 2% isoflurane for a long duration (e.g., 6 h) induces caspase-3 activation and cell death[32]. These results suggest that isoflurane-induced mitochondrial dysfunction (especially mPTP opening) might precede isoflurane-induced cytotoxicity. For the outcomes of H4 naïve cells, 50 μmol/L ginsenoside Rg1 treated for 12 h did not attenuate caspase-3 activation or ATP level, but the change in mPTP had already occurred, whereas extending the treatment to 24 h induced changes in caspase-3 activation and ATP levels. It is interesting that pretreatment with 50 μmol/L ginsenoside Rg1 for 12 h have different effects on isoflurane-induced caspase-3 activation in H4-APP and H4 naïve cells, so 50 μmol/L ginsenoside

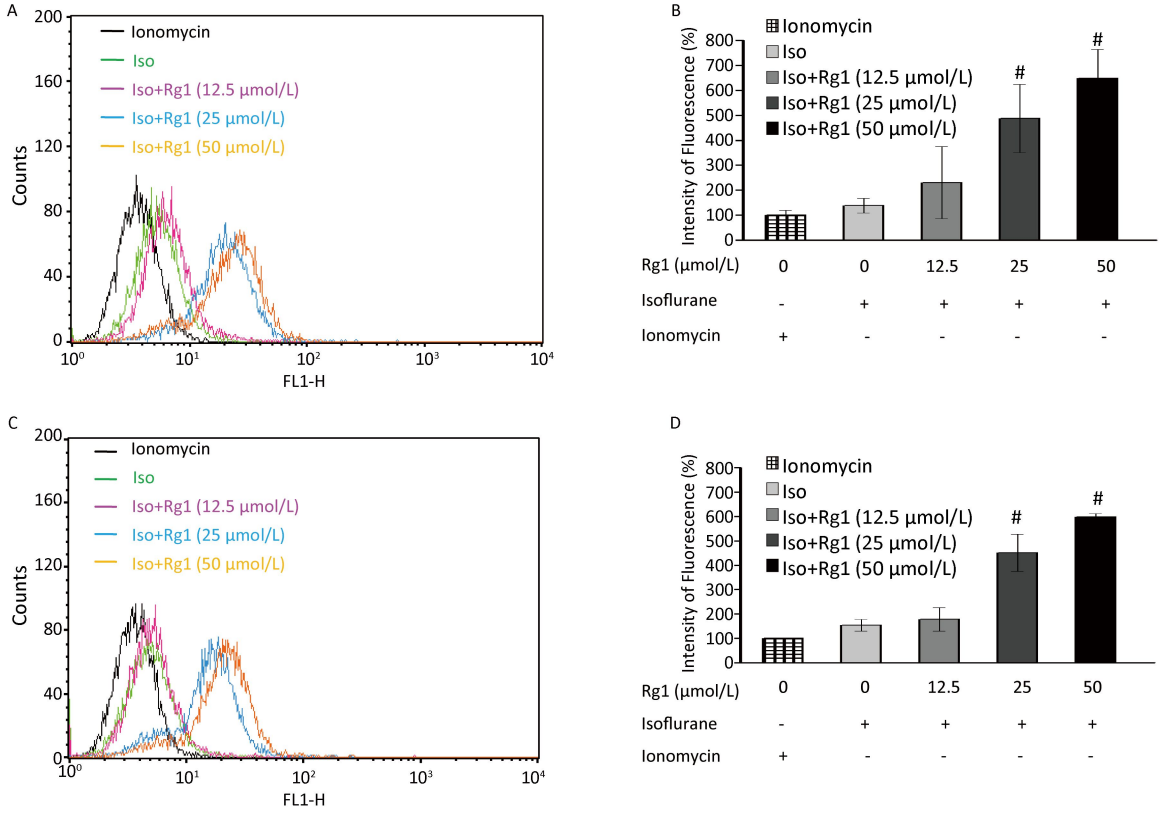

Figure 6. Effect of pretreatment with ginsenoside Rg1 for 24h on isoflurane-induced mPTP opening in H4-APP and H4 naïve cells. (A) Flow cytometry shows changes in H4-APP cells. Peak 1: treatment with ionomycin; Peak 2: treatment with isoflurane; Peak 3: treatment with isoflurane plus 12.5 μmol/L Rg1; Peak 4: treatment with isoflurane plus 25 μmol/L Rg1; Peak 5: treatment with isoflurane plus 50 μmol/L Rg1. Treatment with 25 and 50 μmol/L Rg1 attenuates isoflurane-induced opening of mPTP in H4-APP cells, as demonstrated by the right shift of the curve. (B) Quantification of fluorescence intensity shows that 25 and 50 μmol/L ginsenoside Rg1 (Bars 4 and 5) lead to reductions in isoflurane-induced mPTP opening (Bar 2) in H4-APP cells. (C) Flow cytometry shows changes in H4 naïve cells. Peak 1: treatment with ionomycin; Peak 2: treatment with isoflurane; Peak 3: treatment with isoflurane plus 12.5 μmol/L Rg1; Peak 4: treatment with isoflurane plus 25 μmol/L Rg1; Peak 5: treatment with isoflurane plus 50μmol/L Rg1. Treatment with 25 and 50 μmol/L Rg1 attenuates isoflurane-induced opening of mPTP in H4 naïve cells, as demonstrated by the right shift of the curve. (D) Quantification of fluorescence intensity shows that 25 and 50 μmol/L Rg1 (Bars 4 and 5) lead to reductions in isoflurane-induced mPTP opening (Bar 2) in H4 naïve cells. #versus isoflurane treatment group, $P<0.05$.

Rg1 for 12 h may only attenuate the isoflurane induced caspase-3 activation when levels of Aβ are already elevated (H4-APP cells). In our study, ginsenoside Rg1 treatment resulted in dose- and time-dependent changes in caspase-3 activation and mitochondrial function. Ginsenoside Rg1 treatment affects both H4 naïve and H4-APP cells, so might affect not only patients with AD or older patients but also all age groups.

The studies have a few limitations. First, we only performed the research *in vitro* without using animal models or clinical trials, and we did not assess whether ginsenoside Rg1 can ameliorate isoflurane-induced learning and memory impairment. However, findings from current studies show that ginsenoside Rg1 inhibited isoflurane-induced mitochondrial dysfunction and neurotoxicity would establish a system for future studies in animals and human beings. Second, in this study, we only measured caspase-3 activation. This is because studies have already shown that isoflurane can induce caspase-3 activation, apoptosis, amyloid β accumulation, and neuroinflammation[23-24,33-34]. In addition, a recent study by Burguillos et al.[7] has shown that caspase activation alone without apoptosis might still contribute to AD neuropathogenesis.

In conclusion, we have found that ginsenoside Rg1 can attenuate the common inhalation anesthetic isoflurane-induce caspase-3 activation *in vitro*. Furthermore, we have found that ginsenoside Rg1 can attenuate isoflurane-induced opening of mPTP and decreases in ATP concentration, and is both dose- and time-dependent.

These findings should lead to additional studies to determine the potential effects of anesthetics on POCD and AD neuropathogenesis, the underlying mechanisms, and strategies for prevention and treatment. This study demonstrates that ginsenoside Rg1, one of the main components of ginseng, has a protective effect in the maintenance of mitochondrial function, in addition to neuronal protection. The detailed effects of Rg1 on isoflurane-induced mitochondrial and neuronal dysfunction require further investigation. Our results provide significant evidence that this ancient herb may indeed hold potential benefit in preventing and treating POCD.

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