Rosuvastatin protects PC12 cells from hypoxia/reoxygenation-induced injury by inhibiting endoplasmic reticulum stress-induced apoptosis

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Abstract. The endoplasmic reticulum stress (ERS) response serves an important role in cerebral ischemia-reperfusion injury (CIRI). However, to the best of the our knowledge, the effect of rosuvastatin on the ERS response in CIRI has not yet been studied. In the present study, the effect of rosuvastatin on cell damage in CIRI was investigated; furthermore, the effect of rosuvastatin on the ERS response was explored. Firstly, a hypoxia/reoxygenation (H/R)-induced cell damage model was established in PC12 cells. Cell viability was subsequently detected by a Cell Counting Kit-8 assay. A lactate dehydrogenase kit was used to detect cytotoxicity. TUNEL assay was then used to measure the extent of cell apoptosis, and western blotting was used to analyze the expression levels of the apoptosis-associated proteins Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-9. In addition, western blotting was used to detect the expression levels of ERS-associated proteins, including phosphorylated (p)-protein kinase R-like endoplasmic reticulum kinase (PERK), p-eukaryotic initiation factor 2α and other proteins. Treatment with rosuvastatin led to an increased activity of H/R-induced PC12 cells and a decrease in their cytotoxicity. Rosuvastatin also led to an inhibition in apoptosis and ERS in H/R-induced PC12 cells. After administration of the ERS response activator thapsigargin (TG), TG was found to reverse the protective effect of rosuvastatin on injury of H/R-induced PC12 cells. Taken together, these findings have shown that rosuvastatin is able to protect PC12 cells from H/R-induced injury via inhibiting ERS-induced apoptosis, providing a strong theoretical basis for the use of rosuvastatin in the clinical treatment of CIRI.

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Introduction

Cerebral ischemia-reperfusion injury (CIRI) refers to an acute cerebral dysfunction that occurs after cerebral ischemia for a certain period of time, followed by the restoration of the infarction-associated blood supply (1). The cure rate associated with CIRI is very low, accompanied by a high disability rate and a high mortality rate; and the prognosis is very poor, with the condition seriously affecting individual's health (2). CIRI is a complex multi-factorial process whose underlying mechanism is poorly understood. The current consensus is that post-ischemia reperfusion causes pathophysiological cascade reactions, including the explosive release of intracellular Ca^{2+} ions, excessive production of reactive oxygen species and neutrophil recruitment, which in turn leads to an acceleration of the inflammatory process, sustains ischemia and causes cell damage (3). The underlying pathological mechanisms include energy metabolism disorder, the inflammatory response, oxidative stress, excitatory amino acid toxicity and apoptosis (4).

Recently, a large number of studies have shown that apoptosis induced by endoplasmic reticulum stress (ERS) fulfills an important role in CIRI (5-7). ERS occupies a central role in CIRI and can initiate neuronal apoptosis (8). Hypoxia, energy depletion, acidosis, damage to Ca^{2+} homeostasis and the generation of a large number of free radicals caused by ischemia and reperfusion can all induce ERS, and stress-protective effects in cells may be generated in cells by ERS itself. However, excessive or persistent ERS is able to activate apoptotic signaling pathways, induce apoptosis and aggravate CIRI (9,10). A previously published study reported that the ER-associated protein reticulon 1 is able to mediate CIRI through ERS and mitochondria-associated apoptotic pathways (11). In addition, a vascular endothelial growth factor antagonist was demonstrated to attenuate ischemic/reperfusion (I/R)-induced injury through inhibiting ERS-mediated apoptosis (12). Therefore, an increasing body of evidence supports that ERS has an important role in CIRI.

Among the therapeutic drugs that are currently available for cerebral infarction, statins are often used for primary and secondary prevention, as these have been shown to be important drugs for the treatment of cerebral infarction and to significantly decrease the risk of patients with cerebral infarction (13). In
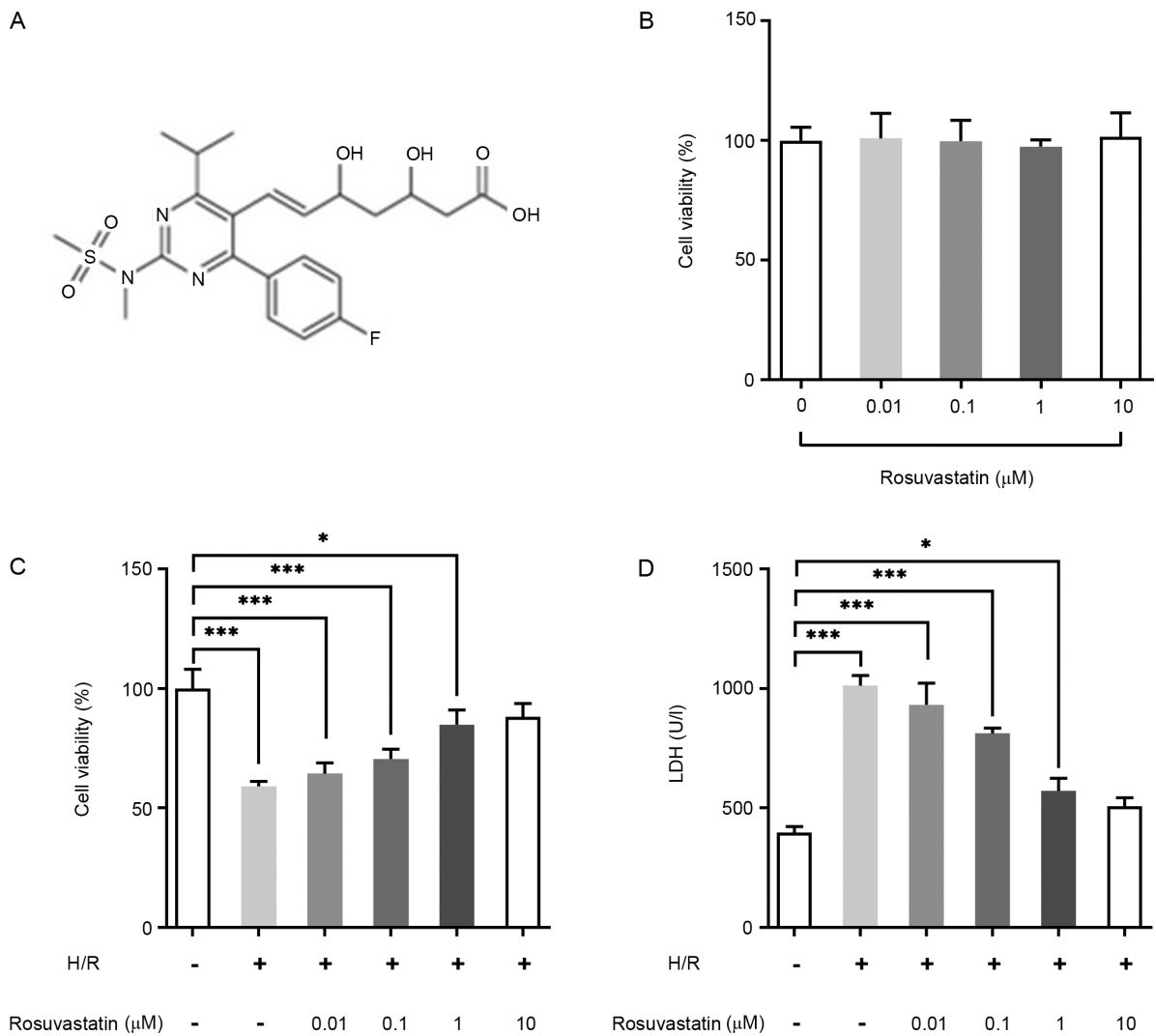
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Figure 1. Rosuvastatin inhibits decreased cellular activity in H/R-induced PC12 cells. (A) Chemical formula of rosuvastatin. (B) CCK-8 detected the cell viability of PC12 cells after induction with different concentrations of rosuvastatin. (C) CCK-8 detected the cell viability of H/R-induced PC12 cells after induction with different concentrations of rosuvastatin. (D) LDH kit detected the cytotoxicity. *P<0.05; ***P<0.001. CCK-8, Cell Counting Kit-8; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase.

addition to its powerful lipid-lowering effects, rosuvastatin is also useful in terms of its anti-inflammatory, antioxidant and immunological regulation effects, and for its ability to improve vascular endothelial function (14). A previous study revealed that rosuvastatin in combination with resveratrol is able to protect the synergic nerve in cases of CIRI (15). Rosuvastatin preconditioning exerts a marked protective effect on focal CIRI in rats, and its mechanism of action is predominantly to decrease oxidative stress and the inflammatory response (16). Rosuvastatin in combination with other drugs has been shown to decrease the size of cerebral infarction (17). In addition, it has been reported in other diseases that rosuvastatin may protect umbilical venous endothelial cells from damage induced under high-glucose conditions by decreasing ERS (18). Rosuvastatin was also shown to inhibit the apoptosis of endothelial cells in ApoE$^{-/-}$ mice via inhibiting ERS, and through the alleviation of atherosclerosis (19). However, to the best of our knowledge, the effect of rosuvastatin on ERS in CIRI has not yet been reported.

In the present study, PC12 cells were induced to establish a cell damage model by hypoxia/reoxygenation (H/R); the apoptosis of the model cells was subsequently detected after administering rosuvastatin, and the underlying mechanism was investigated.

Materials and methods

Cell culture and model induction. PC12 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The PC12 cells were cultured at 37˚C in an atmosphere containing 5% CO_2 in a humidified incubator. For induction of the H/R model, after culture at 37˚C with 5% CO_2 for 24 h, PC12 cells were placed under hypoxic conditions (3% O_2/5% CO_2/92% N_2) for 2 h. The cells were then cultured under normal conditions for 12 h. In terms of cell administration, PC12 cells were pretreated with different concentrations of rosuvastatin (0.01, 0.1, 1 and 10 μm, cat. no. HY-17504A, MedChemExpress) for 12 h at 37˚C before model induction.
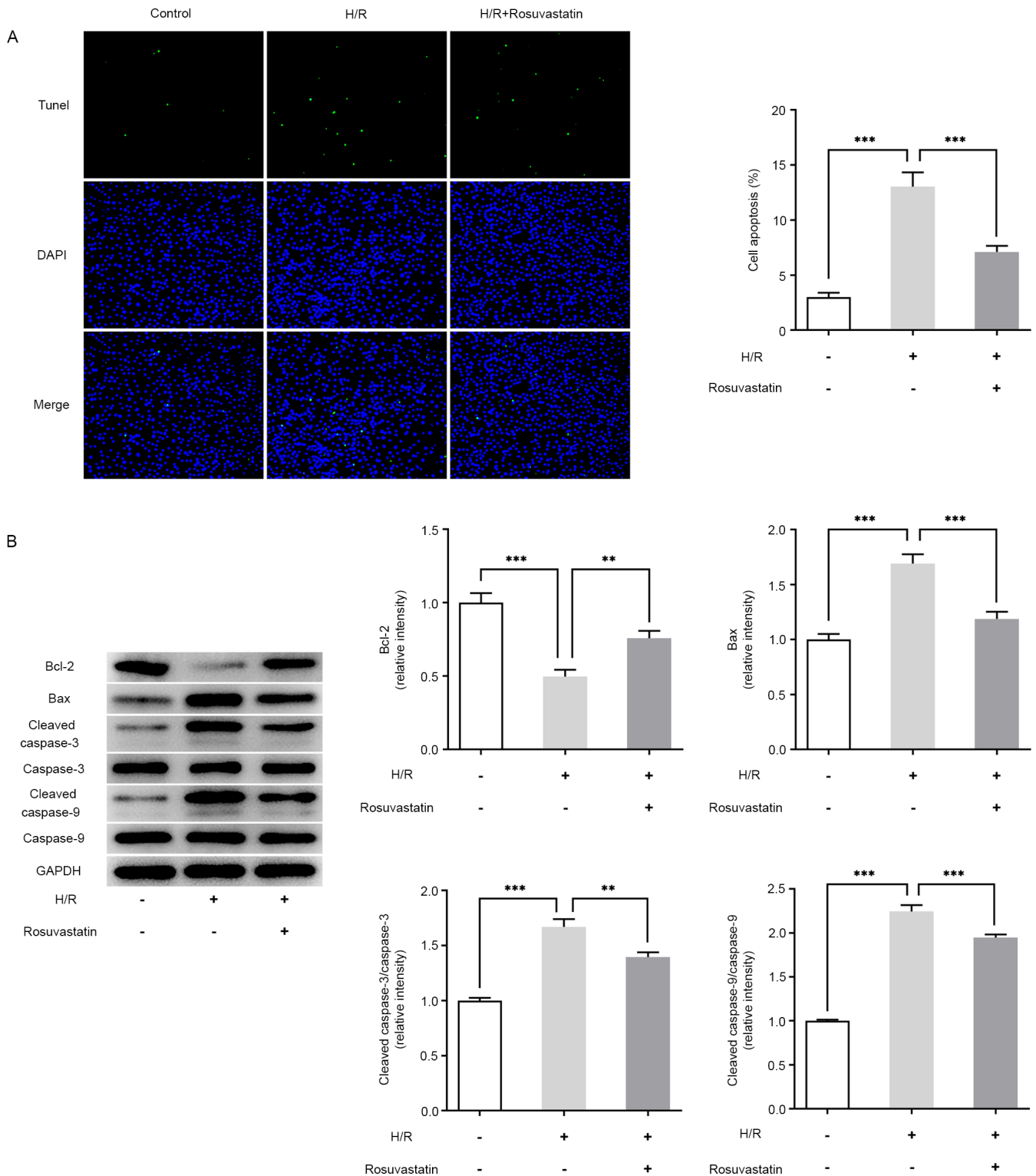
Figure 2. Rosuvastatin inhibits apoptosis of H/R-induced PC12 cells. (A) TUNEL assay detected cell apoptosis. Magnification, x200. (B) Western blotting detected apoptosis-associated proteins. **P<0.01; ***P<0.001. H/R, hypoxia/reoxygenation.

Cell viability assay. Cell viability was measured using a Cell Counting Kit (CCK)-8 assay according to the manufacturer's instructions (Merck KGaA). Briefly, PC12 cells were seeded into 96-well plates (1x10⁴ cells/well). After the cells had reached 80% confluence, they were treated accordingly, and CCK-8 solution (10 μl) was subsequently added to each well for an additional 4 h at 37°C. The absorbance at 450 nm was measured with a microplate reader (Biotek Instruments, Inc.). Cell viability was expressed as a percentage of the control group cells.

Lactate dehydrogenase (LDH) release assay. LDH detection is usually performed for evaluating cytotoxicity. A commercial LDH assay kit was purchased from Nanjing Jiancheng
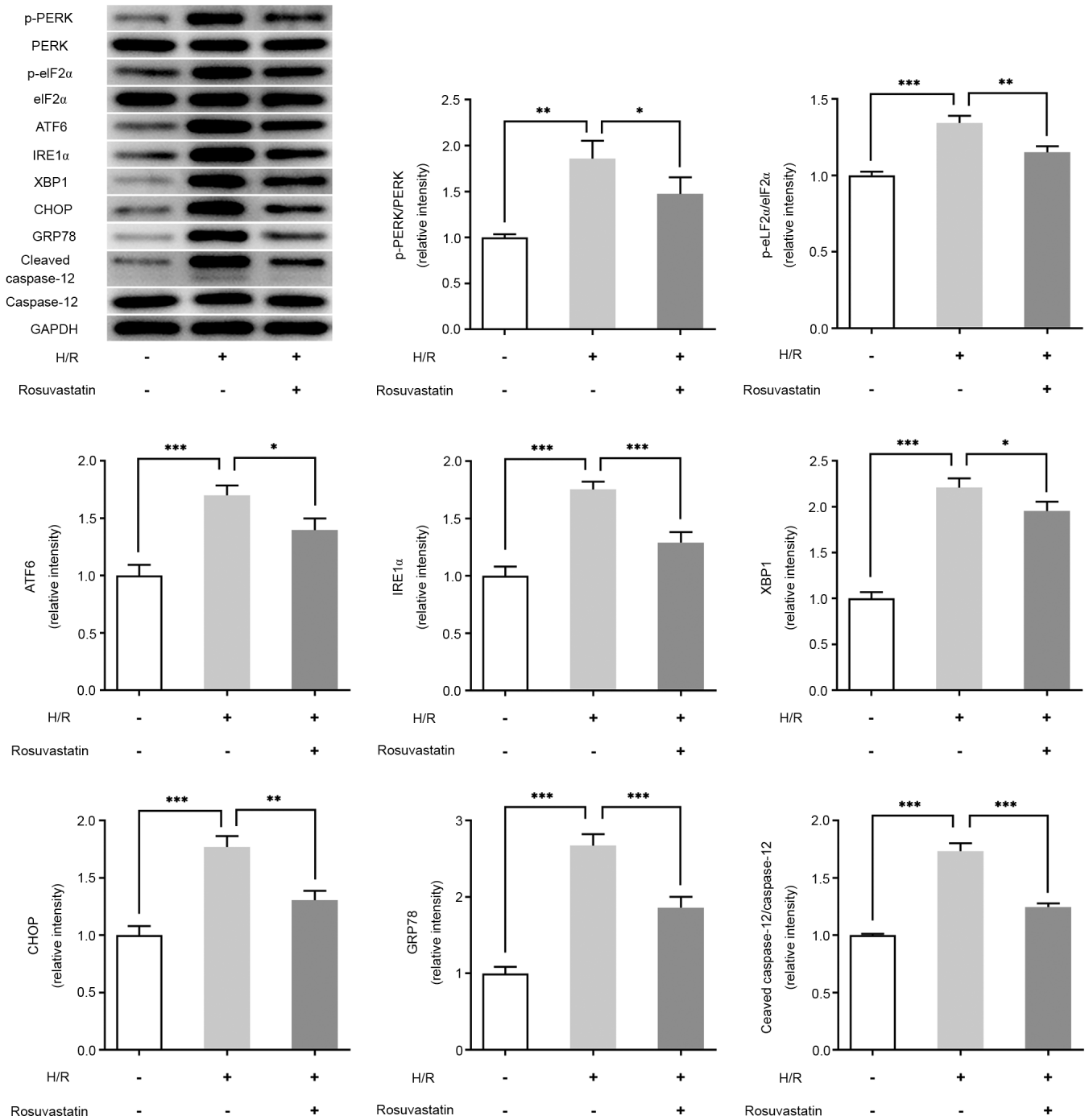
Figure 3. Rosuvastatin inhibits ERS levels in H/R-induced PC12 cells. Western blotting detected ERS-associated proteins. *P<0.05; **P<0.01; ***P<0.001. H/R, hypoxia/reoxygenation; ERS, endoplasmic reticulum stress; p-, phosphorylated-; CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor 2α; ATF6, activating transcription factor 6; IRE1α, inositol-requiring transmembrane kinase/endoribonuclease 1α; XBP1, X box binding protein 1.

Bioengineering Institute (Nanjing, China) and the LDH assay was performed strictly according to the manufacturer's instructions. The absorbance of each sample was detected at 440 nm with a microplate reader. The percentage of cell death was calculated according to the following formula: Viability (%)=(OD$_{Treatment}$-OD$_{Treatment\ blank}$)/(OD$_{Max\ LDH\ activity}$-OD$_{Max\ LDH\ activity\ blank}$)x100%.

TUNEL assay. The extent of apoptosis was detected by TUNEL assay (Promega Corporation), according to the manufacturer's instructions. The PC12 cells (1x10^4 cells/well) were incubated in 6-well plates for 24 h. Cells were subsequently fixed using 4% paraformaldehyde for 30 min at 37˚C, and washed three times with cold PBS. The TUNEL reaction mix was added to the cells and incubated at 37˚C for 1 h. Cells were subsequently rinsed with PBS, streptavidin-HRP was added and the mixture was incubated for 1 h in the dark. Finally, DAB solution was added to develop the color reaction. Cells were observed under a light microscope (magnification, x200), and images were captured. The number of apoptotic cells was calculated as the mean proportion of positive cells out of the total number of cells in six fields of view per slide.
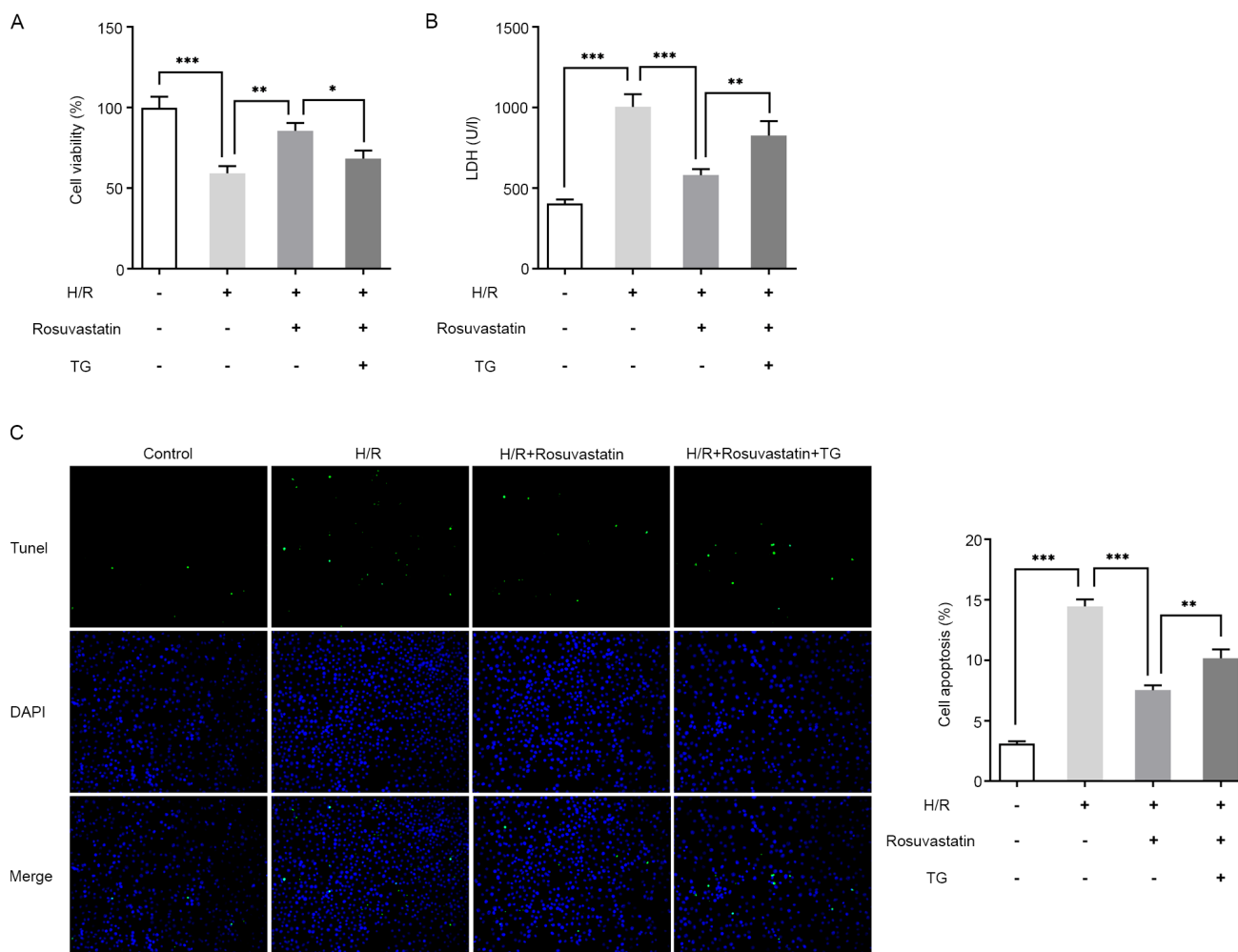
Figure 4. TG reverses the protective effect of rosuvastatin on the cell viability of H/R-induced PC12 cell. (A) Cell Cycle Kit-8 detected cell viability. (B) LDH kit detected cytotoxicity. (C) TUNEL assay detected cell apoptosis. Magnification, x200. *P<0.05; **P<0.01; ***P<0.001. TG, thapsigargin; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase.

Western blot analysis. PC12 cells were washed three times with ice-cold phosphate buffered saline, and lysis buffer (Merck KGaA) was added to the cells for 30 min to isolate the total protein. Protein concentration was determined using a bicinchoninic acid assay protein assay kit (Bio-Rad Laboratories, Inc.). Proteins (25 μg/lane) were separated using 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane, prior to blocking with 5% non-fat milk in Tris-buffered saline/0.10% Tween-20 at room temperature for 2 h. The membranes were then incubated with the following primary antibodies at 4˚C overnight (all antibodies were purchased from Abcam): Anti-Bcl-2 (1:1,000; cat. no. ab182858), anti-Bax (1:1,000; cat. no. ab182733), anti-cleaved caspase-3 (1:1,000; cat. no. ab32042), anti-cleaved caspase-9 (1:1,000; cat. no. ab2324), anti-caspase-3 (1:1,000; cat. no. ab32351), anti-caspase-9 (1:1,000; cat. no. ab32539), anti-phosphorylated (p)-protein kinase R-like endoplasmic reticulum kinase (PERK; 1:1,000; cat. no. ab192591), anti-p-eukaryotic initiation factor 2α (eIF2α; 1:1,000; cat. no. ab32157), anti-eIF2α (1:1,000; cat. no. ab169528), anti-PERK (1:1,000; cat. no. ab229912), anti-GRP78 (1:1,000; cat. no. ab21685), anti-activating transcription factor 6 (ATF6; 1:1,000; cat. no. ab227830), anti-inositol-requiring transmembrane kinase/endoribonuclease

1α (IRE1α; 1:1,000; cat. no. ab37073), anti-X-box binding protein 1 (XBP1; 1:1,000; cat no. ab37152), anti-C/EBP homologous protein (CHOP; 1:1,000; cat. no. ab11419) and anti-GAPDH (1:1,000; cat. no. ab8245), and anti-cleaved caspase-12/caspase 12 (1:1,000; cat. no. #2202; Cell Signaling Technology, Inc.). After washing with Tris-buffered saline/0.10% Tween-20, the blots were incubated with Goat Anti-Mouse IgG H&L (Alexa Fluor® 488; 1:5,000; cat. no. ab150113; Abcam) for 2 h at room temperature. Signals were visualized with enhanced chemiluminescence (Beyotime Institute of Biotechnology), and ImageJ software (Version1.8.0; National Institutes of Health) was used for semi-quantification of the blots.

Statistical analysis. Data are expressed as the mean ± SD. Comparisons among multiple groups were analyzed using a one-way ANOVA followed by a Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Rosuvastatin inhibits decreased cellular activity in H/R-induced PC12 cells. The chemical structure of rosuvastatin is shown in Fig. 1A. PC12 cells were induced by
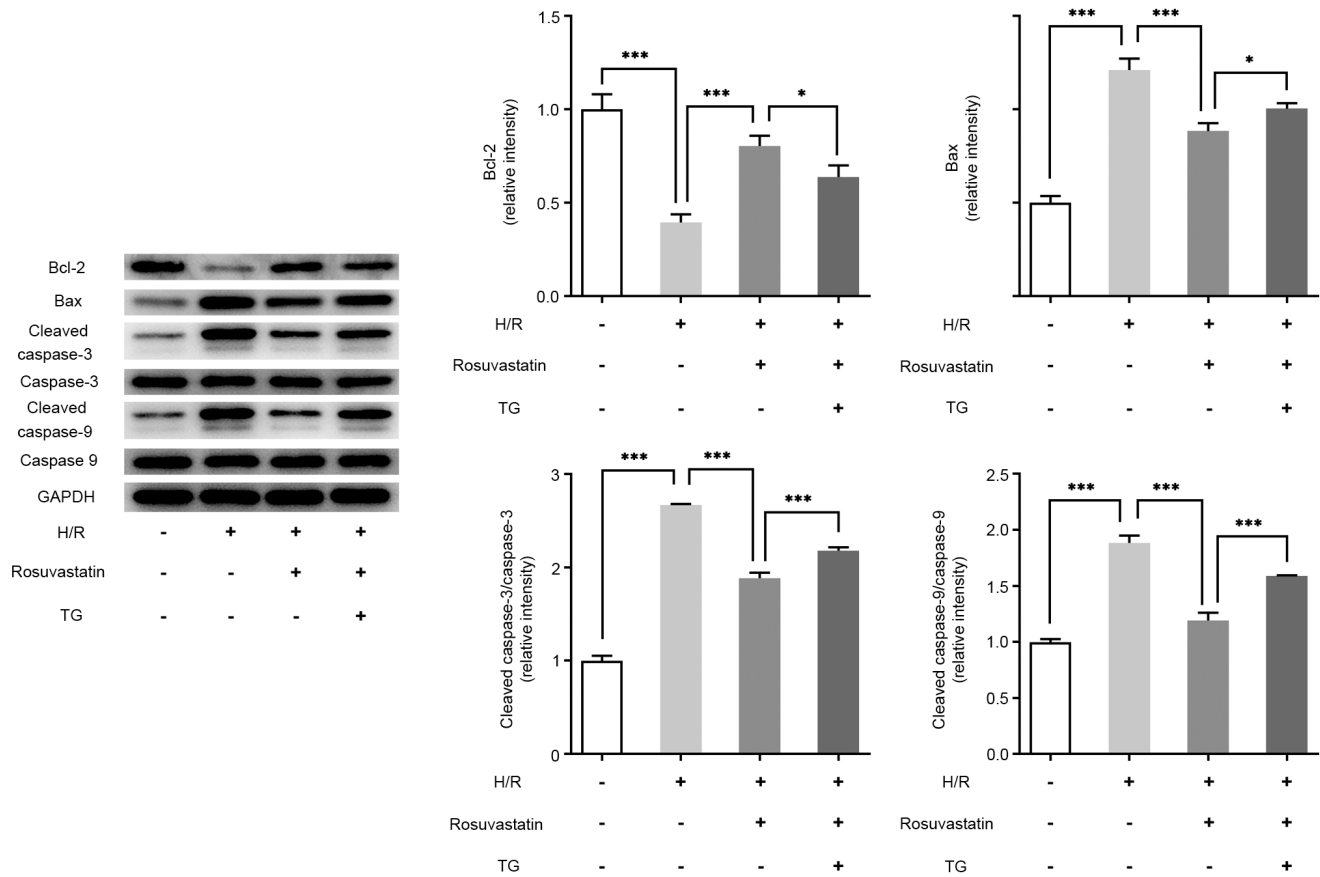
Figure 5. TG reverses the protective effect of rosuvastatin on the apoptosis of H/R-induced PC12 cell. Western blotting detected the apoptosis-associated proteins. *P<0.05; ***P<0.001. TG, thapsigargin; H/R, hypoxia/reoxygenation.

rosuvastatin at different concentrations (0, 0.01, 0.1, 1 and 10 μM). The results of the CCK-8 assay demonstrated that rosuvastatin exerted no significant effect on the viability of PC12 cells (Fig. 1B). Subsequently, CCK-8 assay was used to investigate the effect of rosuvastatin on the viability of H/R-induced PC12 cells, and it was observed that, with an increase in rosuvastatin concentration, the viability of H/R-induced PC12 cells was significantly increased (Fig. 1C). LDH kit assay was then performed for the detection of cytotoxicity, and these results showed that rosuvastatin led to a marked decrease in the cytotoxicity of H/R-induced PC12 cells (Fig. 1D). Taken together, the results from these assays revealed that rosuvastatin treatment led to decreased cellular activity in H/R-induced PC12 cells. In addition, 1 μM Rosuvastatin had significant effects on cell viability and cell LDH release, thus, 1 μM rosuvastatin was selected as the concentration for subsequent experiments.

Rosuvastatin inhibits apoptosis of H/R-induced PC12 cells. Apoptosis was detected using a TUNEL assay. Compared with the control group, apoptosis was found to be significantly increased following H/R induction (Fig. 2A), accompanied by an increased expression of the Bax, cleaved caspase-3, cleaved caspase-9 proteins, whereas the expression level of the Bcl-2 protein was decreased (Fig. 2B). Compared with the H/R group, the apoptotic rate of the H/R + rosuvastatin group was decreased, accompanied by a decreased expression of cleaved caspase-3, cleaved caspase-9, and an increased expression of

Bcl-2. These results showed that rosuvastatin is able to inhibit apoptosis in H/R-induced PC12 cells.

Rosuvastatin inhibits ERS levels in H/R-induced PC12 cells. In order to detect the effect of rosuvastatin on ERS levels in H/R-induced PC12 cells, western blot analysis was used to investigate the expression levels of ERS-associated proteins. Compared with the control group, the expression levels of p-PERK, p-eIF2α, ATF6, IRE1α, XBP1, CHOP, GRP78 and cleaved caspase-12 were significantly increased in the H/R group. Further administration of rosuvastatin, however, reversed the expression levels of p-PERK, p-eIF2α, ATF6, IRE1α, XBP1, CHOP, GRP78, CHOP and cleaved caspase-12 (Fig. 3).

TG reverses the protective effects of rosuvastatin on H/R-induced PC12 cell injury. Subsequently, the mechanism of rosuvastatin action on H/R-induced PC12 cells by adding the ERS activator, thapsigargin (TG), was investigated. To this end, the cells were divided into control, H/R, H/R + rosuvastatin and H/R + rosuvastatin + TG groups. The results of CCK-8 assay showed that, compared with the H/R + rosuvastatin group, the cell survival rate was significantly reversed following TG addition (Fig. 4A). The results of the LDH kit assay revealed that, compared with the H/R + rosuvastatin group, the cytotoxicity of the H/R + rosuvastatin + TG group was significantly increased (Fig. 4B). The results from the TUNEL assay experiments revealed that TG administration was able
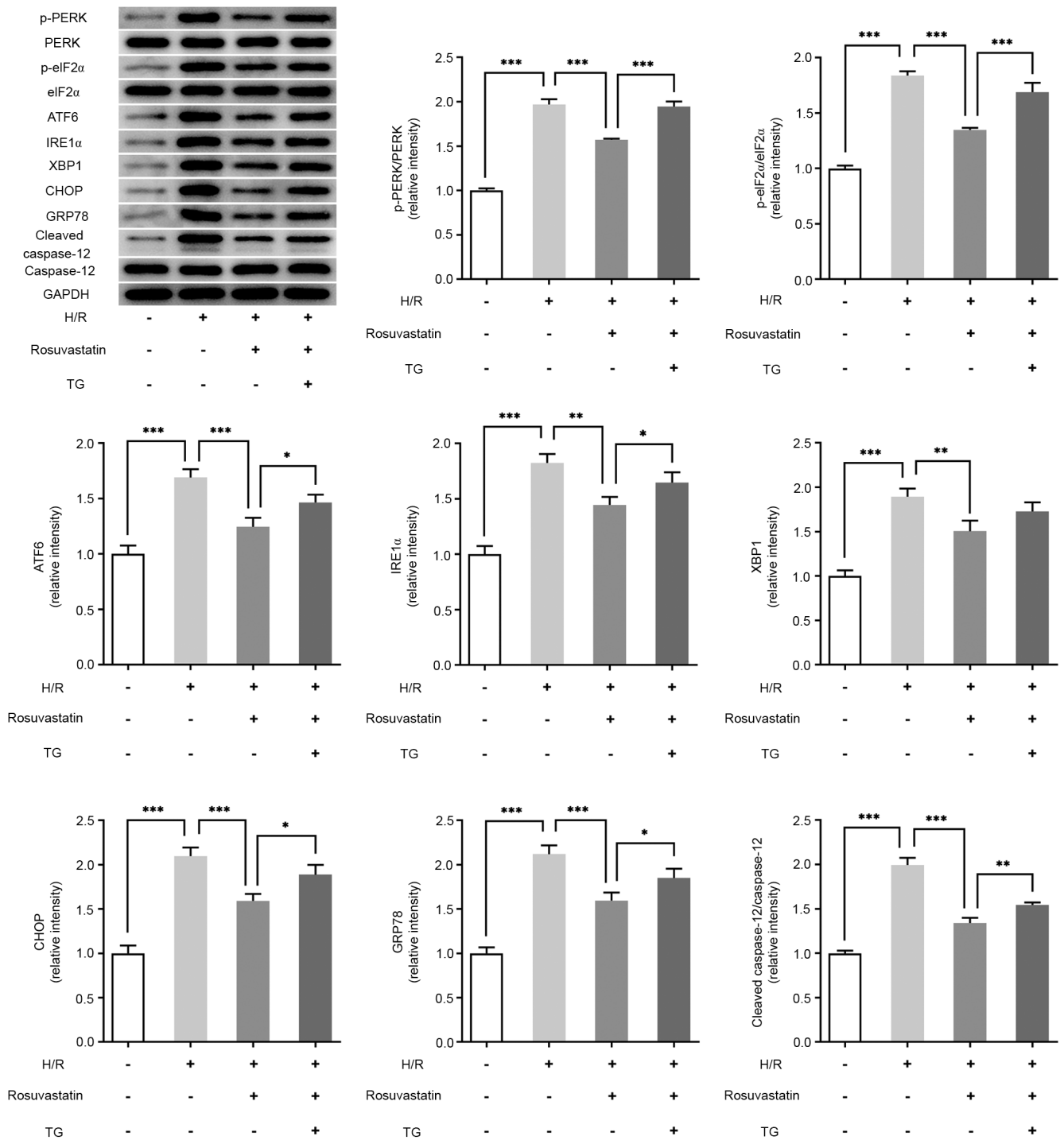
Figure 6. TG reverses the protective effect of rosuvastatin on ERS of H/R-induced PC12 cell. Western blotting detected the ERS-associated proteins. *P<0.05; **P<0.01; ***P<0.001. H/R, hypoxia/reoxygenation; ERS, endoplasmic reticulum stress; p-, phosphorylated-; CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor 2α; ATF6, activating transcription factor 6; IRE1α, inositol requiring transmembrane kinase/endoribonuclease 1α; XBP1, X-box binding protein 1.

to significantly reverse the apoptotic rate of H/R-induced PC12 cells inhibited by rosuvastatin (Fig. 4C). Subsequently, the expression levels of apoptosis-associated proteins were investigated, and the trend in expression level changes was consistent with those of the TUNEL assay (Fig. 5). In addition, the expression levels of ERS-associated proteins were also investigated, and these experiments showed that, compared with the H/R + rosuvastatin group, the expression levels of p-PERK, p-eIF2α, ATF6, IRE1α, XBP1, CHOP, GRP78 and cleaved caspase-12 were significantly increased (Fig. 6). Taken together, the aforementioned experimental results showed that TG reversed the protective effect of rosuvastatin on H/R-induced PC12 cell injury.

Discussion

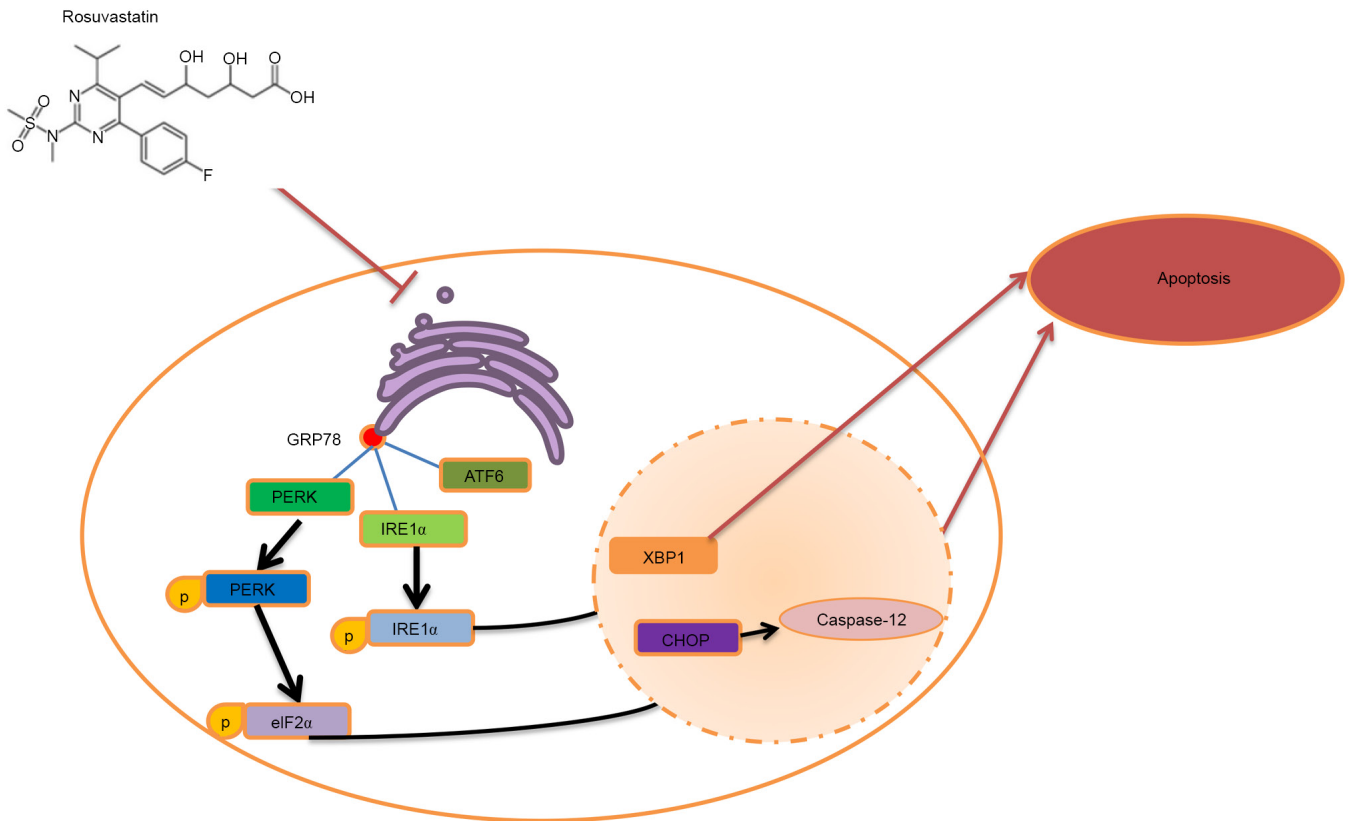PC12 cells are a differentiated cell line of pheochromocytoma in the adrenal medulla of rats. PC12 cells have the

Figure 7. Mechanism of action. p-, phosphorylated-; CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor 2α; ATF6, activating transcription factor 6; IRE1α, inositol requiring transmembrane kinase/endoribonuclease 1α; XBP1, X-box binding protein 1.

general characteristics of neuroendocrine cells and are widely used in neurophysiological and pharmacological studies due to their ability of passage. In addition, a study has used hypoxia-reoxygenation-induced PC12 cells to form a model of cerebral ischemia reperfusion injury model, which has been recognized as a model (20). Therefore, in the present study, PC12 cells were induced to establish a damage model through H/R and carried out relevant experiments.

ERS mediates three different signaling pathways at both the transcriptional and translational levels, predominantly through ER transmembrane proteins, namely, the three unfolded protein response signaling receptor molecules, IRE-1, PERK and activating transcription factor 6 (21). These three signaling molecules are normally inactive. When ERS occurs, heat shock protein family A (Hsp70) member 5 (GRP78) is isolated from these signal receptor molecules, which assists in the process of enabling unfolded or misfolded proteins to recover their correct conformation, and activating these signal receptor molecules, affecting the downstream CHOP levels (22). Previous studies have shown that the inhibition of ERS-mediated apoptosis leads to a decrease in CIRI-induced injury (12,23,24). Therefore, the ER should serve an important role in the development of CIRI. In the present study, a damage model of PC12 cells induced by H/R was established. It was found that the expression levels of ERS-associated proteins were significantly increased, and the cell apoptotic rate was also increased, indicating that ERS-induced apoptosis has an important role in H/R-induced cell damage.

A previous study reported that rosuvastatin is able to alleviate myocardial IR injury by upregulating miR-17-3s-mediated autophagy (25). Rosuvastatin protects isolated hearts from IR injury through the Akt/GSK-3β signaling axis, the metabolic environment and mitochondrial permeability transition hole (26). Pretreatment with rosuvastatin has been shown to decrease oxidative stress and the inflammatory response associated with focal CIRI in rats (16). In addition, rosuvastatin has been demonstrated to have an important neuroprotective role in animal models of CIRI through decreasing inflammatory damage, regulating thrombosis and improving endothelial cell function. Furthermore, rosuvastatin has been widely recommended for primary and secondary defense treatment of CIRI (27-29).

Rosuvastatin alleviates endothelial cell apoptosis and atherosclerosis of ApoE$^{-/-}$ mice by inhibiting ERS (19). It has also been shown that rosuvastatin may protect endothelial cells from hyperglycemia-induced damage by decreasing ERS (18). Rosuvastatin and simvastatin attenuate cisplatin-induced cardiotoxicity through the disruption of ERS-mediated apoptotic death in rats via targeting the ER-chaperone GRP78 and calpain-1 pathways (30). However, the regulatory effect of rosuvastatin on ERS in CIRI has not yet been reported, to the best of our knowledge. In the present study, it was shown that rosuvastatin could inhibit the cell activity decline, decrease the cytotoxicity and inhibit the apoptotic rate and ERS level of H/R-induced PC12 cells. By adding the ERS activator TG, the protective effect of rosuvastatin on H/R-induced PC12 cell injury was found

to be significantly reversed. The aforementioned findings suggested that rosuvastatin may protect PC12 cells from H/R-induced injury through inhibiting apoptosis induced by ERS.

In conclusion, the present study has shown that rosuvastatin protects PC12 cells from H/R-induced injury by inhibiting ERS-induced apoptosis (Fig. 7). The findings may provide a theoretical basis for the clinical treatment of CIRI with rosuvastatin.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ and YG wrote the manuscript and analyzed the data. LL and JY performed the experiments and supervised the study. YG searched the literature and revised the manuscript for important intellectual content. ZZ and YG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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