Effects of *Salidroside* on Cobalt Chloride-Induced Hypoxia Damage and mTOR Signaling Repression in PC12 Cells

Xiaoyong Zhong,^{a,b,d} Ruhui Lin,^{c,d} Zuanfang Li,^d Jingjie Mao,^d and Lidian Chen^{*a,b,c}

^a College of Rehabilitation Medicine, Fujian University of Traditional Chinese Medicine; ^b Key Laboratory of TCM Rehabilitation of State Administration of Traditional Chinese Medicine, Fujian University of Traditional Chinese Medicine; ^c MOE Key Laboratory of Traditional Chinese Medicine on Osteology & Traumatology and Exercise Rehabilitation, Fujian University of Traditional Chinese Medicine; and ^d Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine; 1 Huatuo Road, Minhou Shangjie, Fuzhou, Fujian 350122, P.R. China. Received January 29, 2014; accepted April 27, 2014

Salidroside (SA), a phenylpropanoid glycoside isolated from *Rhodiola rosea* L., has been documented to exert a broad spectrum of pharmacological properties, including protective effects against neuronal death induced by various stresses. To provide further insights into the neuroprotective functions of SA, this study examined whether SA can attenuate cobalt chloride (CoCl₂)-induced hypoxia damage and mammalian target of rapamycin (mTOR) signaling repression in PC12 differentiated cells. Differentiated PC12 cells were exposed to CoCl₂ for 12 h to mimic hypoxic/ischemic conditions and treated with SA at the same time, followed by electron microscopy and analysis of cell viability, intracellular reactive oxygen species (ROS) level, hypoxia-inducible factor-1 α (HIF-1 α) level, and the regulated in development and DNA damage responses (REDD1)/mTOR/ p70 ribosomal S6 kinase (p70S6K) signaling pathway. Our data indicated that SA can dramatically attenuate the ultrastructural damage of mitochondria induced by CoCl₂ and significantly decrease CoCl₂-induced ROS production. Moreover, phosphorylated mammalian target of rapamycin (p-mTOR) was significantly reduced by CoCl₂, and this inhibition was relieved by the treatment of SA in PC12 cells, as evidenced by immunoblot and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses. The SA effects were blocked by pretreatment of RAD001. The results indicate that SA can rescue CoCl₂-induced repression of REDD1/mTOR/ p70S6K signal transduction in PC12 cells. Our data demonstrate that SA is able to attenuate CoCl₂-induced hypoxia damage and mTOR signaling repression, suggesting that SA may protect brain neurons from ischemic injury through mTOR signaling, and provide new insights into the prevention and treatment of cerebral ischemic.

Key words salidroside; ischemic; mammalian target of rapamycin (mTOR); hypoxia; cobalt chloride; PC12 cell

Focal cerebral ischemia or stroke is characterized by obstruction of blood flow to the brain, resulting in deficient supply of oxygen, glucose, serum, and nutrient that are indispensable for the energy generation. Since central nervous system is most susceptible to hypoxia conditions, the continuous oxygen and energy deprivation results in cognitive disturbances and decreased motor control, leading to fainting, long term loss of consciousness, coma, seizures, cessation of brain stem reflexes, and brain death. Ischemic stroke is currently a leading cause of disability and mortality in the aged population, due to limited medication and therapy, and new therapeutic strategies for this devastating disease are urgently needed.

Recently, the role of mammalian target of rapamycin (mTOR) signaling in neurodegenerative and cerebrovascular diseases has attracted great attention.¹⁾ As a serine/threonine kinase, mTOR activation can mediate broad biological activities that include translation initiation, transcription, cytoskeleton organization, cell growth, and proliferation as well as cell survival.²⁾ Based on the ability of mTOR to prevent neuronal apoptosis, inhibit autophagic cell death, promote neurogenesis, and improve angiogenesis, mTOR may acquire the capability of limiting the ischemic neuronal death and promoting the neurological recovery.¹⁾ Consequently, regulation of mTOR activity has emerged as a potential therapeutic strategy for ischemic stroke. It has been shown that mTOR activity is repressed by hypoxia,³⁾ and the mTOR signaling repression is

maintained by the hypoxia-inducible factor-1 α (HIF-1 α), which up-regulates regulated in development and DNA damage responses (REDD1) in a number of cell lines including PC12 cells in response to hypoxia.⁴⁾ And REDD1 is essential for the inhibition of mTOR signaling pathway.^{5,6)}

Cobalt chloride (CoCl₂) is well known as a hypoxia-inducing agent to mimic hypoxic/ischemic conditions, including generation of reactive oxygen species (ROS) and up-regulation of hypoxic-specific genes such as the hypoxia-inducible transcription factor-1 α (HIF-1 α).^{7,8)} The rat pheochromocytoma cell line PC-12 is catecholaminergic, excitable, and widely used for cell signaling and neurochemical studies.^{9,10)} In this study, we find that PC12 cells provide a useful *in vitro* system for the study of hypoxia-induced neuronal injury upon exposure to CoCl₂.^{11,12)}

Salidroside (SA), a natural compound from *Rhodiola rosea* L., is a major active ingredient responsible for most pharmacological effects of *Rhodiola*, such as anti-oxidative, anti-aging, anti-inflammatory, anti-cancer, anti-fatigue and anti-depressant activities.^{13–19)} Increasing evidence suggests that SA may have neuroprotective effects in injured brain. SA can reduce the degree of cerebral edema and the brain infarct size of rats with global cerebral ischemia, relieve the metabolism abnormality of free radicals and improve the function of cognition as well as behavioral and histological outcomes.^{20–22)} Furthermore, several *in vitro* studies have shown that SA protects against neuronal apoptotic death induced by various stimuli, such as glutamate, H₂O₂, hypoglycemia/serum limitation, MPP⁺, and

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: cld@fjtcu.edu.cn

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CoCl₂, via antioxidative action or upregulation of survival signals, such as the Bcl-2/Bax ratio, Akt phosphorylation, and maintenance of mitochondrial integrity.^{23–33} In addition, it has been reported that SA may regulate mTOR signaling in cultured human umbilical vein endothelial cells (HUVECs) or in bladder cancer cell lines.^{34,35} However, whether the neuroprotective effects of SA involve regulation of the mTOR signaling pathway in PC12 cells is still unknown.

To gain further insights into the neuroprotective functions of SA, we have used the PC12 cell model and investigated the underlying mechanism by which SA may protect the cells from CoCl₂-induced damage.

MATERIALS AND METHODS

Materials SA (purity >99%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Mouse 2.5S nerve growth factor (NGF) was purchased from Promega (Madison, WI, U.S.A.). Cobalt Chloride (CoCl₂), 4',6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and horse serum were purchased from Life Technologies (Gaithersburg, MD, U.S.A.). The bicinchoninic acid (BCA) protein assay kit, ROS assay kit was obtained from Beyotime Institute of Biotechnology (Shanghai, China). HIF-1 α antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-mTOR, anti-phospho-mTOR, anti-p70 S6 Kinase (49D7), anti-Phospho-p70 S6 Kinase (Thr389) Rabbit mAb antibodies, rabbit-immunoglobulin G (IgG) conjugated peroxidase and everolimus (RAD001) were purchased from Cell Signaling Technology (St. Louis, MO, U.S.A.). REDD1 Rabbit polyclonal antibody were purchased from Proteintech Europe Ltd. (Manchester, U.K.).

Cell Culture and Treatment PC12 cells were maintained as monolayer cultures in 25-cm² tissue culture flasks in complete medium, CM (DMEM supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin), in a humidified 5% CO₂ incubator at 37°C. Cells were subcultured either in 96-well plates at a density of 1 $\times$ 10⁴ cells/well for cell viability assay, in 6-well plates (1.6 $\times$ 10⁵ cells/well) for ROS determination, or in 100 mm diameter dish (8 $\times$ 10⁵ cells/dish) for all other experiments. PC12 cells were differentiated for 2 d before the treatment with SA or CoCl₂ in low serum medium (LSM) (DMEM supplemented with 1% horse serum, 100 U/mL penicillin, 100 μ g/mL streptomycin), in the presence of 50 ng/mL NGF, followed by various functional tests in the differentiated cells as described below.

Measurement of Cell Viability Cell viability was measured using the MTT assay. CoCl₂ was applied at various concentrations (100, 200, 300, 400, 500, 600, 700 μ M) for 12, 24, and 36 h to investigate the effective concentrations of CoCl₂ for induction of neurotoxicity. After the treatment, 10 μ L MTT solution (5 mg/mL in phosphate buffered saline (PBS)) was added to each well, the plates were incubated at 37°C for 4 h, the culture medium containing MTT was removed and 100 μ L dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan. The absorbance was measured at 570 nm

using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek, Model EXL800, U.S.A.).

With the appropriate concentration of CoCl₂, differentiated PC12 cells were subjected to SA (30, 60, 90, 120 μ M) treatment followed by the MTT assay described above, we determined the effective concentrations of SA and CoCl₂ as 90 μ M and 400 μ M, respectively, which were used in the following experiments.

Electron Microscopy Cells were fixed in 2.5% glutaraldehyde in PBS (pH 7.3). Following treatment in 1% osmium tetroxide solution (OsO₄), 2% uranylacetate (UA) and dehydration in ethanol and acetone series, the samples were embedded in epoxy resin and polymerized 48 h at 60°C. Ultrathin sections were made with a glass knife on an ultramicrotome and picked up on copper net (100 mesh). Sections on grids were post-stained for 2 min with 1% UA and 6 min with 1% lead citrate by floating them on single drops of the staining solution at room temperature, then rinsed in deionized water and dried. Sections were observed by using a H7650 electron microscope (Hitachi, Tokyo, Japan).

Measurement of Intracellular ROS The ROS level was determined with a ROS assay kit (Beyotime, Shanghai, China). In brief, after cell treatments for 12 h, as indicated, cells were incubated with 20 μ M dichloro-dihydro-fluorescein diacetate (DCFH-DA) at 37°C for 30 min in the dark, and then gently rinsed with DMEM 3 times. The fluorescence of DCFH-DA was excited at 480 nm and detected at 530 nm by flow cytometry (FACScalibur, BD Bioscience, San Jose, CA, U.S.A.).

mRNA Quantitation by Quantitative Polymerase Chain Reaction (PCR) Analysis Total RNAs were extracted with TRIZOL[®] Reagent (Invitrogen, Barcelona, Spain), and mRNAs were transcribed to cDNAs using the PrimeScript[®] RT reagent kit with gDNA Eraser (TaKaRa) and used as template for the quantitative PCR assay (Kit SYBR[®] Premix Ex Taq[™], TaKaRa BIO Inc.). PCR was performed in a 7500 fast Real-Time system (ABI, CA, U.S.A.) and the results were analyzed with Software provided by 7500 fast system. Data were normalized with P0 levels and expressed as the ratio between hypoxia and control. The primers used in these reactions are listed in Table 1.⁴⁾

Western Blot Analysis Cell lysates (30 μ L) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 120 min in 5% w/v bovine serum albumin (BSA) at room temperature and then incubated with monoclonal antibodies for the indicated proteins or for β -actin as a loading control over night at 4°C. After washing in TBS with 0.25% Tween-20 (TBST), membranes were incubated

Table 1. Primer Used for the Quantitative PCR

Target	Name	Sequence
P0	5' P0	5'-CCTCATATCCGGGGAATGTG-3'
	3' P0	5'-GCAGCAGCTGGCACCTTATTG-3'
HIF-1 α	5' HIF-1 α	5'-AGTGTACCCTAACTAGCCG-3'
	3' HIF-1 α	5'-TTCACAAATCAGACCAAGC-3'
REDD1	5' REDD1	5'-TCTGGACCCAGTCTAGTGC-3'
	3' REDD1	5'-ACGAGGACCAAGGAAGAGT-3'

with the horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature and the membranes were washed again in TBST. The specific protein bands were analyzed with enhanced ECL Plus reagents (Beyotime, Shanghai, China) and scanned with a Storm PhosphorImager (Bio-Rad Chemi Doc XRS+, U.S.A.).

Statistical Analysis All results were the means of triplicate samples and the data were analyzed using the SPSS package for Windows (Version 16.0). Statistical analysis of the data was performed with Student's *t*-test and ANOVA. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Protective Effect of SA on CoCl_2 -Induced Loss of Cell Viability MTT assay indicated that exposure of PC12 cells to CoCl_2 at different concentrations (100, 200, 300, 400, 500, 600, 700 μM) for 12, 24, and 36 h caused significant decreases in cell viability, and the cell viability loss exhibited a dose-

dependent manner (Fig. 1b). Exposure to 400 μM CoCl_2 for 12 h resulted in nearly 50% decrease in cell viability ($54.8 \pm 9.4\%$ viable cells) as compared to untreated control cells (100% viability). So in the following experiments, the 400 μM CoCl_2 treatment for 12 h was used to mimic hypoxic/ischemic conditions in PC12 cells. We also found that different treatment of SA (30, 60, 90, 120 μM) was able to protect cells from CoCl_2 -induced cell viability loss, with maximum protection (79.6 ± 14.4 viable cells) at 90 μM (Fig. 1c).

SA Blocks Hypoxia-Induced Cell Death upon CoCl_2 Treatment It's well known that CoCl_2 induces apoptosis in PC12 cells through ROS.³⁶⁾ In this regard, the CoCl_2 -induced PC12 cell death was triggered within 12 h with 400 μM CoCl_2 treatment, corresponding to the cell viability loss (Fig. 1b) and morphological changes under light microscopy (date not shown). Furthermore, we used electron microscopy to determine the ultrastructural changes in the cell upon CoCl_2 treatment, the results showed that nucleolus disappeared, condensed chromatin relocalized to the inner side of an in-

Fig. 1. Protective Effect of SA on CoCl_2 -Induced Loss of Cell Viability

(a) Chemical structure of SA (*p*-hydroxyphenethyl- β -D-glucoside). (b) PC12 cells were exposed to CoCl_2 (100, 200, 300, 400, 500, 600, 700 μM) for 12, 24, and 36 h, and the cell viability was measured by MTT assay. (c) MTT assay after different cell treatments, which included exposure to 400 μM CoCl_2 for 12 h without and with treatment of SA at 30, 60, 90, 120 μM for 12 h at same time, PC12 cells undergoing neither SA treatment nor CoCl_2 stimulation served as control. Results are mean $\pm$ S.E.M. of four independent experiments; * $p < 0.05$ by ANOVA. #, &, Δ , $p < 0.05$ indicate differences between different dose treatment of CoCl_2 in 12 h, 24 h, and 36 h, respectively.

tact nuclear membrane, and mitochondria were swollen with disintegration and lysis of the cristae in CoCl_2 -treated PC12 cells in comparison to untreated control cells (Fig. 2b). In this regard, SA significantly alleviated the CoCl_2 -induced ultrastructural changes characteristic of cellular damage (Fig. 2c).

Furthermore, we investigated the effects of SA on CoCl_2 -induced elevation of ROS production in PC12 Cells. Flow cytometry with molecular probe DCFH-DA was used to monitor alterations in the intracellular ROS level. The results were evaluated by relative fluorescence units (RFU). As compared to control, exposure of differentiated PC12 cells to CoCl_2 treatment significantly increased the RFU by 2.40 ± 0.14 fold (Fig. 3b), indicating an elevation of ROS production. SA treatment attenuated the CoCl_2 -induced increase in ROS production, as evidenced by a significant decline in the fluorescence intensity to 1.86 ± 0.04 (Fig. 3b).

The Neuroprotective Mechanism of SA Involves the REDD1/mTOR/p70S6K Signaling Pathway It is well known that hypoxia triggers oxidative stress and down-regulates mTOR signal transduction, including generation of ROS and increased expression of HIF-1 α , REDD1, *etc.*,^{4,7,8,27,36} which represses mTOR signaling in PC12 cells in response

to hypoxia.³⁻⁵ We found a significant increase in the levels of HIF-1 α (0.45 ± 0.02 fold increase) and REDD1 (0.62 ± 0.02 fold increase) (Fig. 4b, c) in the PC12 cells after a 12h treatment by $400 \mu\text{M}$ CoCl_2 , and this up-regulation of HIF-1 α and REDD1 was attenuated by addition of SA ($90 \mu\text{M}$) (Fig. 4b, c). Furthermore, our real-time qRT-PCR analysis showed that CoCl_2 treatment increased the mRNA level of REDD1 but not HIF-1 α level (Figs. 4d, e). In contrast, SA treatment profoundly reduced the mRNA levels of both REDD1 and HIF-1 α . Next we analyzed the phosphorylation status and levels of mTOR, p70S6K (Fig. 5a) by Western blot. As shown in Figs. 5b and c, a 12h CoCl_2 treatment ($400 \mu\text{M}$) showed a significant reduction in the levels of mTOR and p70S6K (0.24 ± 0.01 fold, 0.39 ± 0.1 fold, respectively) compared with untreated control cells, and SA was able to attenuate the repression of mTOR, p70S6K to 0.41 ± 0.02 fold, 0.65 ± 0.06 fold ($p < 0.05$), respectively. However, when the cells were pretreated with 2nM RAD001 (everolimus), an mTOR inhibitor, SA-induced increase in mTOR, p70S6K expression was almost blocked (Fig. 5).

Fig. 2. Effect of SA on CoCl_2 -Induced Cell Damage Analyzed by Electron Microscopy

PC12 cells incubated in the absence (a) of CoCl_2 showed a normal ultrastructural, whereas in the presence (b) of $400 \mu\text{M}$ CoCl_2 for 12h, showed a ultrastructural change with nucleolus disappeared, condensed chromatin localized to the inner side of an intact nuclear membrane, mitochondria (arrows) swollen with disintegration and lysis of cristae in PC12 cells. With the treatment of SA (c), SA group showed a slight apoptosis ultrastructural changes and drastic ultrastructural changes of mitochondrial compared with model group. The arrows indicate the ultrastructural change of mitochondria.

Fig. 3. Effect of SA on CoCl₂-Induced ROS Production in PC12 Cells

ROS production in PC12 cells was measured by flow cytometry following DCFH-DA staining. FCM results of 3 groups were overlaid in one picture (a). The results were evaluated by relative fluorescence units (RFU) (b). Results are mean $\pm$ S.E.M. of three independent experiments; * p < 0.05, compared with the control group (no treatment). ** p < 0.05, compared with the group that treated with CoCl₂ only.

DISCUSSION

Ischemic stroke is one of the major causes of mortality and long-term disability in aged population, and very few treatment options are available.³⁷⁾ As such, developing new neuroprotective drugs and investigating the molecular mechanism of neuroprotection have become a major research focus in the field. SA, as a small-molecule compound of *Rhodiola rosea* L. with a defined chemical structure as phenol glycoside (Fig. 1a), has been documented to possess neuroprotective effects against neuronal damage induced by various insults.^{19–33)} However, the underlying mechanisms are still not well understood.

As a serine/threonine kinase, mTOR plays a central role in translation initiation, transcription, cytoskeleton organization, cell growth, and proliferation as well as cell survival. mTOR functions through mTORC1 and mTORC2 complexes and their multiple downstream substrates, such as eukaryotic initiation factor 4E-binding protein 1 (4EBP1), p70 ribosomal S6 kinase (p70S6K), sterol regulatory element-binding protein 1, serum- and glucocorticoid-induced protein kinase 1, etc.²⁾ The two major well-established downstream targets of mTORC1 are p70S6K and 4EBP1. Active mTORC1 can activate p70S6K and the activation of p70S6K promotes mRNA biogenesis, translation of ribosomal proteins, and cell growth.³⁸⁾ A large body of studies have revealed the neuroprotective effects of mTOR activity during cerebral ischemia. Administration of estradiol to adult female ovariectomized rats prior to focal cerebral ischemia significantly reduces infarct volumes and decreases apoptosis in the cerebral cortex and concurrently prevents ischemia induced-decrease in the expression of phosphorylated mTOR and p70S6K.³⁹⁾ Depletion of S6K1 enhances oxygen glucose deprivation (OGD), an *in vitro* model of ischemia, induced injury in astrocytes. In contrast, repletion of S6K1 expression by adenoviral infection reduces cell injury.⁴⁰⁾ Moreover, the absence of S6K expression in the brain increases infarct volume and mortality in mice follow-

ing focal cerebral ischemia.⁴⁰⁾ In addition, inhibition of mTOR activity by rapamycin decreases cell survival and increases apoptotic injury in neurons and microglia during OGD.^{41–43)} Erythropoietin, the hematopoietic growth factor and neuron-protectant, has been shown to protect microglia against OGD through enhancing mTOR activity and preventing mitochondrial cytochrome *c* release, since inhibition of mTOR by using rapamycin abrogates the cytoprotection of erythropoietin.⁴⁴⁾ Rapamycin can also increase the brain infarct size as well as increase the neurological deficit scores following focal cerebral ischemia in rats.⁴¹⁾ These studies suggest that the mTOR signaling pathway is involved in the underlying neuroprotective mechanisms during cerebral ischemia. As increasing evidence suggests that SA may have neuroprotective effects in injured brain,^{20–22)} we speculate that SA may exert the neuroprotective effects by regulating the mTOR signaling pathway.

In this study, we have shown that SA is capable of protecting PC12 cells against CoCl₂-induced hypoxia damage by maintaining mTOR signaling activity, as evidenced by up-regulated phosphorylation of mTOR, p70S6K. Furthermore, RAD001, also known as Everolimus, an immunosuppressant analog of rapamycin that can dephosphorylate mTOR downstream targets, can block the mTOR signaling activity maintained by SA. It has been shown that mTOR activity is repressed by hypoxia, and the mTOR signaling repression is maintained by HIF-1 α , which up-regulates REDD1 in a number of cell lines including PC12 cells in response to hypoxia.³⁾ In this study, our data show that CoCl₂ can inhibit the activity of mTOR through the up-regulation of REDD1 and HIF-1 α expression, as evidenced by Western-blot results. Although the mRNA level of HIF-1 α does not change with increased protein level, this may be caused by instantaneous induction of HIF-1 α in response to hypoxia,⁴⁵⁾ and posttranscriptional regulation as opposed to transcription initiation.⁴⁶⁾ Taken together, our data suggest that SA attenuates hypoxia-induced repression of mTOR signaling through down-regulating the protein levels of HIF-1 α and REDD1 in PC12 cells. SA, acting as a

Fig. 4. Effect of SA on HIF-1 α and REDD1 Expression in PC12 Cells

HIF-1 α and REDD1 protein levels (a) measured by Western blot analysis. The results were evaluated by the ratio of densitometric unit and β -actin was used as loading control (b, c). Data are representatives of three independent experiments. mRNA level of HIF-1 α and REDD1 were determined by real-time PCR analysis (d, e). Data are normalized relative to P0 mRNA levels and the value for the control was considered as 1. Each experiment was done in triplicate twice and data corresponding to the mean $\pm$ S.E.M. of RNA from three different extractions. * $p < 0.05$, compared with the control group (no treatment). ** $p < 0.05$, compared with the group that treated with CoCl₂ only.

neuroprotective drug, may limit ischemic neuronal death and promote neurological recovery *via* sustaining mTOR signal transduction. Interestingly, Liu *et al.*³⁵⁾ reported that SA can decrease the growth of bladder cancer cell lines *via* inhibition of the mTOR pathway. As the mTOR pathway regulates many major cellular processes and is implicated in an increasing number of pathological conditions, including cancer, obesity, type 2 diabetes, and neurodegeneration,³⁸⁾ we speculate that SA may exert a unique dual effect, protective for normal cells but toxic to tumor cells.

Additionally, our flow cytometry and electron microscopy data show that CoCl₂ strongly stimulates ROS production in PC12 cells and causes drastic ultrastructural changes including swollen mitochondria with disintegration and lysis of cristae, which is consistent with the results by Zou *et al.*³⁶⁾ Of note, our present study showed that SA can significantly

attenuate the CoCl₂-induced ultrastructural damage of mitochondria and ROS production. REDD1 is suggested to functions as a direct regulator of mitochondrial metabolism, and mediates a negative feedback pathway to HIF-1 α through regulation of ROS.^{34,47–50)} The drastic ultrastructural changes of mitochondria suggest that SA may act as a neuroprotective agent as well as a antioxidant to attenuate the CoCl₂-induced mitochondrial dysfunction and ROS production, which in turn change the HIF-1 α stabilization and REDD1 expression, further sustaining mTOR signal transduction. Moreover, considering the drastic ultrastructural changes of mitochondria but not much decrease in ROS level in SA treatment group, we speculate that SA may play a more important role in neuroprotective effects than anti-oxidative effects during cerebral ischemia.

In conclusion, our results demonstrate that SA is able to at-

Fig. 5. Effect of SA on mTOR Signaling in CoCl₂-Induced PC12 Cells

PC12 cells were pretreated with 2 nM RAD001 for 2 h before being exposed to 400 μM CoCl₂ for an additional 12 h. Phosphorylation status and levels of mTOR, p70S6K, were measured by Western blot (a). The results were evaluated by the ratio of densitometric unit (b, c). Data are representatives of three independent experiments. **p* < 0.05, compared with the control group (no treatment). ***p* < 0.05, compared with the group that treated with CoCl₂ only. ****p* < 0.05, compared with the group that treated with CoCl₂ and SA.

tenuate CoCl₂-induced hypoxia damage and mTOR signaling repression in PC12 cells, suggesting that SA may protect brain neurons from ischemic injury through sustaining mTOR activity, and provide a useful approach in prevention and treatment of ischemic stroke.

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