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Salvianolic acid A protects human SH-SY5Y neuroblastoma cells against H_2O_2-induced injury by increasing stress tolerance ability

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ABSTRACT

Salvianolic acid A (Sal A) is a polyphenol extracted from the root of the *Salvia miltiorrhiza* bunge. Hydrogen peroxide (H_2O_2) is a major reactive oxygen species (ROS), which has been implicated in stroke and other neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease. In this study, we investigated the neuroprotective effects of Sal A in human SH-SY5Y neuroblastoma cells against H_2O_2-induced injury. Our results showed that cells pretreated with Sal A exhibited enhanced neuronal survival and that this protection was associated with an increase in adenosine triphosphate (ATP) and the stabilization of mitochondrial membrane potential. In addition, Sal A markedly decreased the excessive activation AMP-activated protein kinase (AMPK) and the serine–threonine protein kinase, Akt, in SH-SY5Ycells induced by H_2O_2. In conclusion, our results demonstrated that Sal A protects SH-SY5Y cells against H_2O_2-induced oxidative stress and these protective effects are related to stress tolerance and not energy depletion via inhibition of the AMPK and Akt signaling pathway.

1. Introduction

Salvianolic acid A (Sal A) is a polyphenol extracted from the root of *Salvia miltiorrhiza* bunge (also known as Danshen), which has been used for treatment of ischemic cardiocerebral vascular diseases thousands of years in China [1]. Growing evidence suggests that Sal A may account for the cardiocerebral protective properties of Danshen [2–6]. Recently, several molecular mechanisms of Sal A pharmacological activity have been investigated. Currently, several studies have revealed that Sal A plays an important role in a variety of cellular activities, including potent antioxidant activity [7], anti-apoptotic activity [8], improving rCBF [9] and anti-inflammatory effects [10], among others. Sal A has also been shown to function as a natural inhibitor of protein–protein interactions mediated by Src-family SH2 domains [11]. Moreover, Sal A can inhibit platelet activation and arterial thrombosis via inhibition of phosphoinositide 3-kinase [12]. Furthermore, Sal A suppresses the lipopolysaccharide-induced NF-kappaB signaling pathway by targeting IKKbeta [10]. Although our understanding of the role of Sal A in several diseases has grown over the past few years, further insight into the effects and mechanisms of Sal A is needed to utilize Danshen's therapeutic potential to treat ischemic cardiocerebral vascular diseases.

Reactive oxygen species (ROS) have been hypothesized to play an important role in the coordinated cellular signaling [13]. The H_2O_2 ROS plays a crucial role during neuronal cell death in stroke and other neurodegenerative conditions [7,14]. Human SH-SY5Y neuroblastoma cells are widely used as an experimental cell system for the study of neuronal cell damage [14,15]. In this study, we evaluated H_2O_2-induced damage to human SH-SY5Y neuroblastoma cells to examine the protective effects of Sal A.

Abbreviations: Sal A, Salvianolic acid A; H_2O_2, hydrogen peroxide; ATP, adenosine triphosphate; AMPK, AMP-activated protein kinase; rCBF, regional cerebral blood flow; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; PKB, protein kinase B; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenylte-trazoliumbromide; RLU, luminance; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing 0.05% Tween-20; PDGF-BB, platelet-derived growth factor BB.

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The AMP-activated protein kinase (AMPK), a serine threonine kinase, is a key metabolic and stress sensor/effector [16]. Growing evidence indicates that the serine–threonine protein kinase, Akt (also referred to as protein kinase B, PKB), is an important mediator of cell survival. Several reports indicate that Akt plays a key role in a broad spectrum of vital cellular functions [17,18]; thus, it is important to analyze the role of Akt in a given model and environment. However, the effects of Sal A on AMPK and Akt levels in neurocyte damage have not yet been reported.

Sal A possesses one of the most important cardiocerebral protective properties of Danshen. We investigated the neuroprotective effects mechanisms of Sal A, with regard to cell viability, ATP content, mitochondrial membrane potential and the expression of AMPK and Akt during H_2O_2-induced injury in SH-SY5Y cells.

2. Materials and methods

2.1. Cell culture and treatments

Human SH-SY5Y neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and F-12 (GIBCO, Gaithersburg) supplemented with 10% fetal bovine serum (FBS, Hyclone) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The media was replaced every 2 days. Prior to the experiments, the SH-SY5Y cells were plated in 96-well plates at a density of approximately 1.5×10^4 cells per well (for MTT, ATP and mitochondrial membrane potential assays) and in 6-well plates at 8×10^6 cells per well (all other assays). The cells were then treated with H_2O_2 after 24 h.

To examine the potential toxic effects and to obtain a suitable H_2O_2 concentration, the SH-SY5Y cells were treated with H_2O_2 at concentrations that ranged from 100 to 500 μM for 24 h. From this preliminary experiment, a concentration of 200 μM H_2O_2 was selected to evaluate the neuroprotective effects of Sal A on cell viability. Sal A was added to the medium 30 min prior to treatment with H_2O_2. For a single experiment, each treatment was performed in triplicate.

2.2. Cell viability assays

A cell survival analysis was performed according to the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenylte-trazoliumbromide (MTT, Cell Titer 96® AQueous Cell Proliferation Assay kit, Promega, Madison, WI) assay method. Briefly, the cells were cultured with H_2O_2 (24 h) and Sal A (24.5 h), and 10 μl of 4 mg/ml MTT solution was added to each well of the 96-well plate. The cells were then incubated for 4 h in the dark. Next, the absorbance was measured in a microplate reader at 490 nm, and the results were expressed as a percentage of the control.

2.3. Measurement of ATP levels

The ATP level was measured using a luciferase-coupled ATP quantification assay (CellTiter-Glo Luminescent Cell Viability Assay; Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the cells were cultured with H_2O_2 (24 h) and Sal A (24.5 h), and then equilibrated at room temperature for 30 min. Next, 100 μl of CellTiter-GloTM reagent was added to 100 μl of the cell-containing medium. The contents were then mixed for 2 min to induce cell lysis, and the cells were incubated at room temperature for an additional 10 min to stabilize the luminescent signal. The luminance (RLU) was measured by a monochromatormicroplate reader (SpectraMax M5, Molecular Devices, USA).

2.4. An assessment of the mitochondrial membrane potential

The mitochondrial membrane potential was assessed using the fluorescent dye JC-1 (Beyotime Biotechnology, Jiangsu, China). Briefly, the cells were cultured with H_2O_2 (24 h) and Sal A (24.5 h) and then incubated with the JC-1 staining solution (5 μg/ml) for 20 min at 37 °C. Next, the cells were rinsed twice with JC-1 staining buffer, and the fluorescence intensity of the JC-1 aggregates was detected at an excitation wavelength of 525 nm and an emission wavelength of 590 nm. The JC-1 monomer was measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm using a microplate reader. The fluorescence intensity ratio of aggregates to monomers was calculated as an indicator of the mitochondrial membrane potential.

2.5. Western blotting

We treated SH-SY5Y cells with 200 μM H_2O_2 for 5, 10, 15, 30, 60, 120 and 180 min, and analyzed the cells by western immunoblotting with t-AMPK and p-AMPK antibodies. Next, the effects of Sal A on AMPK and Akt signaling were determined.

Briefly, SDS-PAGE 12% separating gels were run using 20 μg of protein samples. After gel electrophoresis, protein extracts were transferred to polyvinylidene-difluoride membrane and then blocked with 5% BSA in Tris buffered saline (TBS, pH 7.4) containing 0.05% Tween-20 (TBST) for 1 h at room temperature on a shaker platform. Each piece of membrane was then separately incubated with rabbit multiclonal anti-p-AMPK (1:1000, cell signaling), anti-p-Akt (1:1000, cell signaling) and monoclonal anti-GAPDH (1:3000, Imagen Riosciences) overnight at 4 °C in TBST. Secondary antibodies were incubated for 1 h at room temperature (biotinylation-conjugated goat anti-rabbit IgG was used for p-AMPK and p-Akt, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was used for GAPDH, 1:1000, Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD, China). Third antibody (HRP- streptavidin, 1:1000, Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD, China) used for p-AMPK and p-Akt was incubated for 30 min at room temperature. The membranes were visualized using ECL chemiluminescence (Molecular Imager ChemiDoc XRS+ System). The band size and density were quantified using Quantity One software (Bio-Rad).

The membranes incubated with anti-p-AMPK and anti-p-Akt were treated with stripping buffer after detected, which were analyzed with AMPK and Akt antibody, using the same western immunoblotting methods as p-AMPK and p-Akt.

2.6. Statistical analysis

The statistical analysis was performed using a one-way analysis of variance. The data were expressed as the mean ± S.E.M. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. The effects of Sal A on the viability of SH-SY5Y cells exposed to H_2O_2

To determine the optimal concentration of H_2O_2, SH-SY5Y cells were cultured with varying concentrations of H_2O_2 for 24 h. Because our results showed a decreased level of the OD value (by 71%) after treatment with 200 μM of H_2O_2 (Fig. 1A), we selected a 24-h treatment of 200 μM H_2O_2 for subsequent experiments in the present study. As shown in Fig. 1B, the viability of the SH-SY5Y cells exposed to 200 μM H_2O_2 for 24 h was reduced compared with the normal group. In addition, co-treatment with Sal

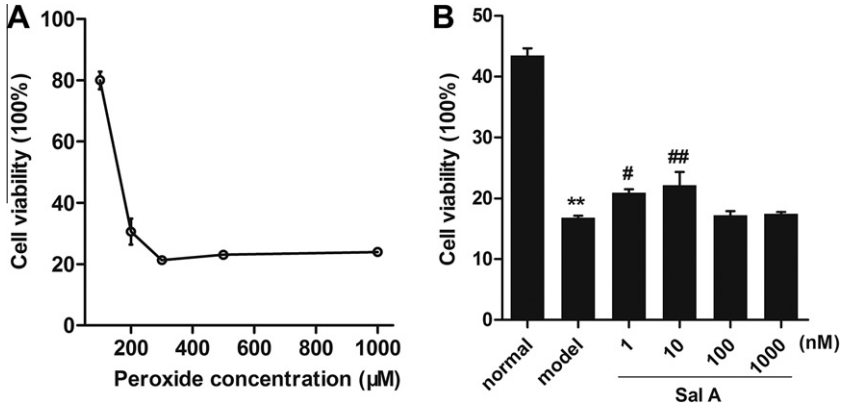

Fig. 1. The effects of Sal A on cell survival against H_2O_2-induced cytotoxicity. SH-SY5Y cells were treated with H_2O_2 at concentrations that ranged from 100 to 500 μM for 24 h. A cell survival analysis was performed using an MTT assay to obtain the optimal H_2O_2 concentration. The SH-SY5Y cells were cultured with 200 μM H_2O_2 for 24 h and Sal A (1, 10, 100 and 1000 nM) for 24.5 h. Next, cell survival analysis was performed. (A) The dose-dependent toxic effects of H_2O_2 on SH-SY5Y cell viability. (B) The effects of Sal A on SH-SY5Y cell viability. The values are expressed as the mean ± S.E.M. ($n = 3$). $^{**}P < 0.05$ compared with the normal group, $^{\#}P < 0.1$, $^{\#\#}P < 0.05$ compared with the model group.

A significantly reduced H_2O_2-induced cell death at 10 nM ($P < 0.05$).

3.2. The effects of Sal A on the energy metabolism of SH-SY5Y cells exposed to H_2O_2

In the present study, Sal A exhibited a significant protective effect on H_2O_2-induced ATP deficiency. Sal A at 10 nM ($P < 0.05$) clearly increased ATP production (Fig. 2A).

3.3. The effects of Sal A on the mitochondrial membrane potential of SH-SY5Y cells exposed to H_2O_2

A mitochondrial probe of JC-1 was used to evaluate the mitochondrial membrane potential. As shown in Fig. 2B, when the SH-SY5Y cells were exposed to 200 μM H_2O_2 for 24 h, the mitochondrial membrane potential was significantly decreased ($P < 0.05$). However, the cells that were preincubated with Sal A (10 nM and 100 nM) prior to the addition of H_2O_2 demonstrated a marked increase in the mitochondrial membrane potential ($P < 0.05$) compared with the H_2O_2-treated cells. These results showed that Sal A suppressed the H_2O_2-induced decrease in the mitochondrial membrane potential.

3.4. The effects of Sal A on AMPK and Akt

3.4.1. Determination of the time-dependent effects of H_2O_2 on AMPK activation in SH-SY5Y cells

As shown in Fig. 3A, a significant increase in AMPK and p-AMPK activity was observed in SH-SY5Y cells that were exposed to 200 μM H_2O_2 between 5 and 120 min. At 180 min, the enhanced levels of p-AMPK had decreased. In contrast, the AMPK expression was still maintained at a high level. Thus, we chose to expose to the cells to 200 μM H_2O_2 for 5 min to investigate the effects of Sal A on AMPK and Akt signaling.

3.4.2. The effects of Sal A on AMPK in SH-SY5Y cells

When the cells were treated with H_2O_2 for 5 min, the expression of AMPK ($P < 0.05$) and p-AMPK ($P < 0.01$) was upregulated (Fig. 3B and C). However, our results revealed that pretreatment with Sal A attenuated the increase of AMPK/GAPDH. Furthermore, pretreatment with Sal A at 1 nM ($P < 0.01$) and 10 nM ($P < 0.05$) significantly attenuated the increase of p-AMPK/AMPK.

3.4.3. Effects of Sal A on Akt in SH-SY5Y cells

When the cells were treated with H_2O_2 for 5 min, the expression of Akt ($P < 0.01$) and p-Akt ($P < 0.05$) was upregulated

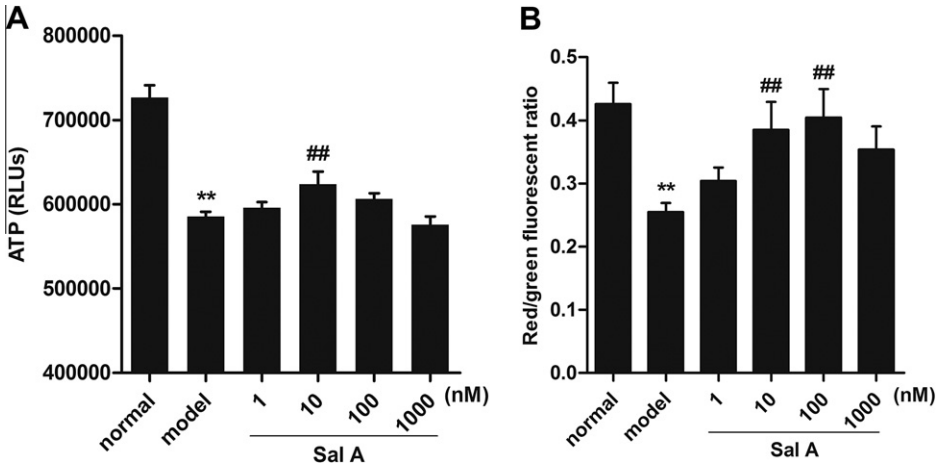

Fig. 2. The effects of Sal A on the ATP content (A) and mitochondrial membrane potential (B) of SH-SY5Y cells. SH-SY5Y cells were treated with H_2O_2 (200 μM) for 24 h and Sal A (1, 10, 100 and 1000 nM) for 24.5 h. Next, the ATP and mitochondrial membrane potential were measured. The values are expressed as the mean ± S.E.M. ($n = 3$). $^{**}P < 0.05$ compared with the normal group, $^{\#\#}P < 0.05$ compared with the model group.

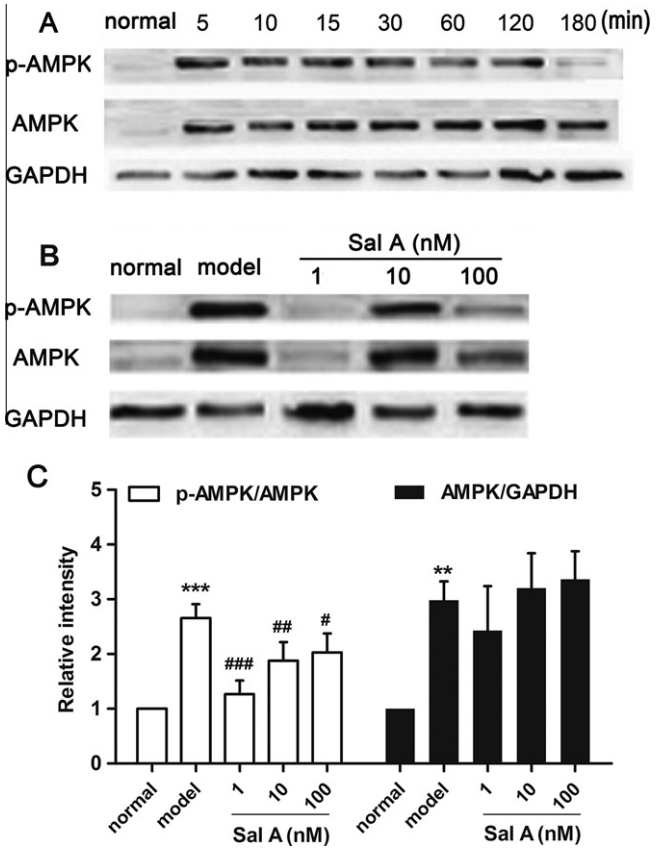

Fig. 3. The effects of Sal A on AMPK levels in SH-SY5Y cells. (A) The time-dependent effects of H_2O_2 on AMPK and p-AMPK in SH-SY5Y cells. The SH-SY5Y cells were treated with H_2O_2 for 5, 10, 15, 30, 60, 120 and 180 min. The AMPK and p-AMPK expression levels were determined by Western blotting ($n = 3$). (B) The effects of Sal A on AMPK and p-AMPK in SH-SY5Y cells. The SH-SY5Y cells were treated with Sal A (1, 10 and 100 nM) for 30 min and treated with 200 μM H_2O_2 for 5 min. The AMPK and p-AMPK expression levels were determined by Western blotting ($n = 3$). (C) The data are expressed as the percentage of the control value at the same time points. The values are expressed as the mean ± S.E.M. $^{**}P < 0.05$, $^{***}P < 0.01$ compared with the normal group, $^{\#}P < 0.1$, $^{\#\#}P < 0.05$, $^{\#\#\#}P < 0.01$ compared with the model group.

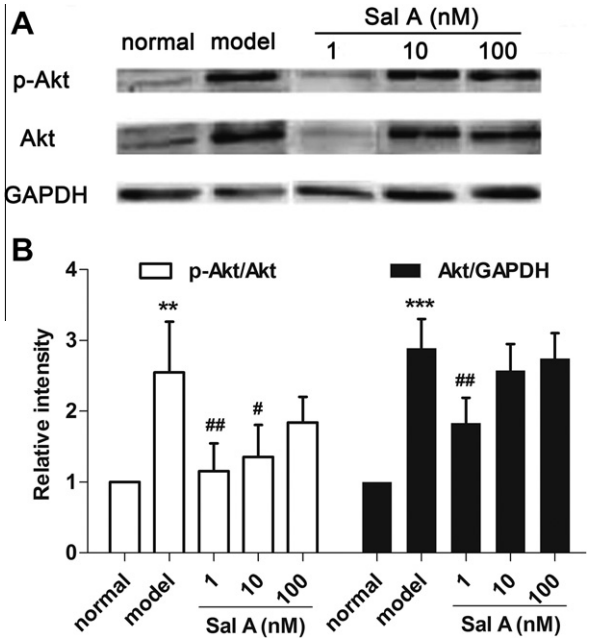

Fig. 4. The effects of Sal A on Akt levels in SH-SY5Y cells. The SH-SY5Y cells were treated with Sal A (1, 10 and 100 nM) for 30 min and treated with 200 μM H_2O_2 for 5 min. The Akt and p-Akt levels were determined by Western blotting ($n = 3$). (A) The effects of Sal A on Akt and p-Akt levels in SH-SY5Y cells. (B) The data are expressed as the percentage of the control value at the same time points. The values are expressed as the mean ± S.E.M. $^{**}P < 0.05$, $^{***}P < 0.01$ compared with the normal group, $^{\#}P < 0.1$, $^{\#\#}P < 0.05$, $^{\#\#\#}P < 0.01$ compared with the model group.

(Fig. 4). However, pretreatment with 1 nM ($P < 0.01$) Sal A attenuated the increase of Akt/GAPDH. Consistent with these results, pretreatment with 1 nM ($P < 0.05$) Sal A significantly attenuated the increase of p-Akt/Akt.

4. Discussion

In this study, we investigated the effects of Sal A on the injury of SH-SY5Y cells induced by H_2O_2. The decreasing survival rates of SH-SY5Y cells induced by H_2O_2 were increased after treatment with Sal A, and this protection was associated with the increased production of ATP and stabilization of the mitochondrial membrane potential. These findings indicated a protective effect of Sal A on H_2O_2-induced injury of SH-SY5Y cells, which is consistent with previous studies [8,19].

Based on previous results, we further examined the effect of Sal A on AMPK in H_2O_2-induced injury of SH-SY5Y cells. AMPK is a well-known sensor of peripheral energy balance and is activated when the cellular energy supply is low, as signaled by increasing intracellular adenosine monophosphate and declining adenosine triphosphate levels. Peripherally, AMPK acutely regulates cellular metabolism, chronically regulates gene expression, reduces energy utilization and increases energy production [20]. The downstream effect of AMPK activation on cell survival is complicated and differs depending on (1) the tissue examined, (2) the degree of stress (mild versus severe), which is related to the timing, duration and amount of AMPK activation and (3) the metabolic capacity of the cells examined. For example, in peripheral organs, such as the heart, activation of AMPK during low-energy states, such as ischemia, can reduce damage [21].

However, whether activation of AMPK is beneficial or detrimental during brain ischemia has engendered considerable controversy. As a major component of the nervous system, neurons display minimal activity of the key glycolytic enzyme, PFK-2 [22] and have a lack of glycogen stores [23]. Thus, neurons are extremely sensitive to hypoxia and hypoglycemia. Previous studies have shown that AMPK may protect neurons against hypoxia, metabolic and excitotoxic insults, amyloid β-peptide and reduced ischemia-induced cerebral injuries [24–26]. Other reports also indicate that AMPK activation may be deleterious. An acute administration of the AMPK activator metformin enhanced metabolic dysfunction and acidosis after stroke and increased infarct size [27]. Recent studies have shown that prolonged AMPK activation enhances the transcriptional activation of the pro-apoptotic factor, Bim, a Bcl-2 family member [28]. In this study, the expression of AMPK and p-AMPK was significantly upregulated in H_2O_2-treated cells, which may be a self-compensatory response from the cell to reduce energy utilization and increase energy production. However, pretreatment with Sal A markedly attenuated the changes in expression of AMPK and p-AMPK induced by H_2O_2. These results implied that excessive AMPK activation in vitro is deleterious to SH-SY5Y cells and that Sal A acts to decrease the excessive activation of AMPK, which results in neuroprotection, by relieving acidosis and decreasing apoptotic activation.

The actions of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway in cerebral ischemia have been widely reported [29–31]. Activation of Akt is extremely important in protecting cells against neuronal cell death. Previous studies have demonstrated that Akt primarily prevents apoptosis via a direct interaction with

the pro-apoptotic molecule, Bad [32], by preventing its homodimerization (and heterodimerization with the anti-apoptotic factor, Bcl-2). In the present study, we observed that Akt and p-Akt levels were significantly increased after treatment with H_2O_2 for 5 min in SH-SY5Y cells, similar to the changes observed in AMPK expression levels. The increased expression of Akt and p-Akt might be a cell compensatory response to stress, which is consistent with previous studies [33,15]. However, in our study, the levels of Akt and p-Akt were significantly decreased when pretreated with Sal A. Lin et al. reported that Sal A suppressed the increase of phosphorylated Akt in HSC-T6 cells enhanced by the platelet-derived growth factor BB (PDGF-BB) [34]. Similarly, Huang et al. reported that similar to the PI3K inhibitors, LY294002 and TGX-221, Sal A potently inhibited PI3K, as shown by a reduction in Akt phosphorylation, which inhibited agonist-induced platelet activation [12]. Taken together, Sal A is thought to decrease the expression levels of Akt and p-Akt. In addition, Hillion et al. provided evidence in a model of ischemic tolerance in PC12 cells that overactivation of Akt was detrimental rather than beneficial. They reported that the protective effects of hibernation, a model of natural tolerance toward cerebral ischemia, were associated with a downregulation of Akt [35]. Taken together, it would be interesting to investigate the detailed mechanisms of the inhibition of Sal A on PI3K/Akt during neural injury in future studies.

Based on previously described results, we propose that activation of AMPK and Akt is a type of compensatory mechanism elicited by the cell to stress, which appears to be a beneficial effect. However, excess activation appears to be detrimental during experimental stroke, while inhibition of this kinase was protective [35,36]. In this study, H_2O_2 toxicity caused the activation of AMPK and Akt in SH-SY5Y cells, while Sal A markedly decreased their activity. Similar to hypothermia and hibernation, our results and that of others demonstrated neuroprotection via inhibition of the excessive activation of AMPK and Akt. Thus, we propose that Sal A contributes to stress tolerance in the cell rather than an energy depletion.

In conclusion, Sal A exhibits a protective effect in H_2O_2-induced injury in SH-SY5Y cells. Stress tolerance via inhibition of AMPK and Akt might be a central mechanism utilized by Sal A to exert neuroprotection against excess stress induced by H_2O_2. Thus, Sal A may be a promising drug candidate for the treatment of diseases involving cerebral stress, such as stroke or other neurodegenerative diseases.

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