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Original article

Protection of Salidroside on Primary Astrocytes from Cell Death by Attenuating Oxidative Stress

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ABSTRACT

Objective To investigate whether salidroside (SAL) has protective and anti-oxidative effects on astrocytes. **Methods** Firstly, SAL was extracted from the roots of *Rhodiola rosea* with 70% ethanol and butanol to obtain crude phenylethyl alcohol glycosides which have been known as bioactive part of *R. rosea*; Secondly, WST-1 assay was carried out to assess the cell viability of astrocytes and cortical neurons under the treatment of the purified (> 95%) SAL. Moreover, WST-1 assay was also used to evaluate the cytoprotective effects of SAL preventing astrocytes from staurosporine-induced cell death; Thirdly, we examined the spontaneous reactive oxygen species (ROS) and staurosporine-induced ROS generation in astrocytes in the absence or presence of SAL. **Results** SAL was observed to improve the astrocytes viability but not cortical neurons. In addition, SAL was able to ameliorate staurosporine-induced cell death. Moreover, SAL was able to attenuate the spontaneous ROS and staurosporine-induced ROS generation. **Conclusion** We here confirm that the anti-oxidative effect of SAL on primary astrocytes might be an important mechanism accounting for the cytoprotective effects from SAL.

Key words

astrocytes; cell death; oxidative stress; reactive oxygen species; salidroside

1. Introduction

Rhodiola rosea L. belongs to genus *Rhodiola* L., subfamily of Sedoideae, family of Crassulaceae. *R. rosea* is an important medicinal plant widely distributed at high altitude in the Arctic and mountainous regions in Europe and Asia, such as in Yunnan, Qinghai provinces and Tibet Autonomous Region, China (Alberdi et al, 2010; Cai et al, 2012). Species in *Rhodiola* L. were used as traditional Tibetan medicines with many medical functions, such as clearing lungs, eliminating toxins from the body, and treating various epidemic diseases, fatigue, traumatic injuries, and burns (Han et al, 2002a; Zuo et al, 2014). On the other hand, the roots of species in *Rhodiola* L. had a long application history in traditional Chinese medicine (TCM) for enhancing human physical and mental performance (Bayliak and Lushchak, 2011), improving cognitive functions (Spasov et al, 2000), reducing mental fatigue (Darbinyan et al, 2000; Shevtsov et al, 2003), promoting free radical mitigation (Wing et al, 2003), and strengthening learning and memory (Abidov et al, 2003; Chen

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et al, 2008; De Bock et al, 2004). Salidroside (SAL), rosavins, and *p*-tyrosol, isolated from the roots of *R. rosea*, were considered as the most important therapeutic components, and they were regarded as the standard to evaluate the quality of plant as well (Kelly, 2001).

Reactive oxygen species (ROS), including superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radical, are regarded as factors involved in fundamental mechanisms of a variety of diseases, such as atherosclerosis, diabetes, cancer, neuro-degeneration, and aging (Ray et al, 2012). Cellular ROS are generated endogenously as in the process of mitochondrial oxidative phosphorylation, or they may arise from the interactions with exogenous sources such as xenobiotic compounds. Oxidative stress occurs when ROS overpower the cellular anti-oxidant defense system, which was indicated by an induction of ROS levels, resulting in a decrease in the cellular anti-oxidant capacity. Oxidative stress led to ROS-mediated damage of nucleic acids, proteins, and lipids, which may occur directly or indirectly. These processes have been implicated in carcinogenesis (Trachootham et al, 2009). ROS regulated several signaling pathways through interaction with critical signaling molecules, affecting a variety of cellular processes, such as cell proliferation, metabolism, differentiation, and cell survival (Ray et al, 2012).

SAL is the major phenylpropanoid glycoside and pharmacologically active ingredient of *R. rosea*. In recent years, it was demonstrated that SAL exhibited many activities, such as anti-aging, anti-cancer, anti-inflammatory, anti-hypoxia, and anti-oxidative properties (Mao et al, 2010; Skopinska-Rozewska et al, 2008; Yu et al, 2007; 2008). Moreover, several studies showed that SAL had the protective effects on neurons (Zhang et al, 2011) and cardiomyocytes (Zhang et al, 2009). However, there were no reports of SAL on assaying its activities in astrocytes, and it remained unclear whether SAL could provide protection against cell death of astrocytes induced by oxidative stress. Astrocytes are demonstrated as the numerous cell types in the central nervous system, playing an important role in providing structural, trophic, and metabolic support to neurons and modulate synaptic activity. Without close communication with astrocytes, neurons can not survive in the brain. As a result, the survival of astrocytes modulates normal brain function. In this study, we are aiming at clarifying whether SAL was able to promote cell viability of astrocytes in the absence and presence of the cell-toxic compound staurosporine (STS). Moreover, we investigated whether SAL was capable of blocking ROS production, either spontaneous or in STS-treated cells.

2. Materials and methods

2.1 Preparation of SAL

The roots of *Rhodiola rosea* L. (8 kg, from Chengdu Herbal Medicine Market, China) were extracted for 4 h with boiling aqueous ethanol (70%) for three times. The solvent was filtered and evaporated in vacuum, and then the concentrated extract was successively partitioned with petroleum ether, ethyl acetate, and *n*-butanol. The *n*-butanol fraction (containing crude phenylethyl alcohol glycosides) was chromatographed repeatedly on silica gel and eluted with $CHCl_3$-CH_3OH (50:0→0:1). The eluent was combined by monitoring with thin-layer chromatography to obtain six fractions (Frs. 1−6). The Fr. 3 was subjected to silica gel and eluted with $CHCl_3$-CH_3OH (8:1) to yield SAL. The chemical structure of SAL was confimed by nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) (Han et al, 2002b; Kasai et al, 1989; Nicholson and Wilson, 1989), and compared with the reference.

2.2 Astrocytes culture

Primary astrocytes-enriched cell cultures were obtained from newborn rats, according to the method described before (Zhu and Reiser, 2014). All procedures using tissue from animals were approved by corresponding regulations from Sachsen-Anhalt, Germany. In brief, newborn rats were decapitated; Total brains were removed and collected in ice-cold Puck's-D1 solution (NaCl, 137 mmol/L; KCl, 5.4 mmol/L; KH_2PO_4, 0.2 mmol/L; Na_2HPO_4, 0.17 mmol/L; glucose, 5.0 mmol/L; sucrose, 58.4 mmol/L, pH 7.4). The brains were gently passed through meshes (256 and 136 μm pore diameter) and centrifuged at 400 *g* for 5 min. The cells were re-suspended in Dulbecco's minimum essential medium (DMEM, Biochrom, Germany) containing 10% heat-inactivated fetal calf serum (FCS, Biochrom, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were plated in the flasks and kept in culture for 10−13 d, and then astrocytes were used for experiments.

As for the experiments, astrocytes were detached by trypsin and re-seeded on 96-well plates at a density of 3×10^4 cells per well. Astrocytes were kept in culture for 24 h before carrying out WST-1 assay or DCF-DA assay. All the cell cultures and sub-cultures were cultured in the humidified incubator with 10% CO_2 at 37 °C. The medium was changed for the first time after 5 d and thereafter every 2 d. For the induction of cell death, astrocytes were treated with 0.2 μmol/L STS in FCS-free DMEM for 24 h.

2.3 Cortical neuronal culture

Primary cortical neurons were obtained from brains of 1−3 day-old rats. The cell suspension was prepared according to the protocol described (Gorbacheva et al, 2013) and cells were plated on dishes coated with ethyleneimine polymer solution (1 mg/mL). The cells were allowed to sediment for 1 h at 37 °C in 5% CO_2, then the floating cells were removed and 1.5 mL culture medium (neurobasal medium A containing 2% supplement B-27 and 0.5 mmol/L *L*-glutamine) was added. Two days later, cytosine arabinoside (AraC, 10^{-5} mol/L) was added to suppress the growth of glial cells. For experiments, neurons were used at days 9−10 in culture. All animal procedures were approved by Ethics Committee of the German Federal State of Sachsen-Anhalt.

2.4 Cell viability

Cell viability was determined using WST-1 assay (Roche, Germany). The reagent WST-1 is a colorimetric assay for the nonradioactive quantification of cellular proliferation, viability, and cytotoxicity. Astrocytes were cultured in 96-well plates (3×10^4 cells/well) containing complete medium overnight. The next day, cells were treated with FCS-free medium for 6 h and cells were incubated with different concentration of SAL (1, 5, 10, 20, and 30 μg/mL) at 37 °C for 48 h. After addition of WST-1 solution (10 μL of WST-1 into 90 μL of FCS-free medium), the cells were subsequently incubated at 37 °C for 1 h. The optical density was read at 450 nm using a microplate reader (Molecular Devices, USA). The experiments were repeated for four times independently.

As for the cellular protection, astrocytes were firstly incubated with 1 μg/mL of SAL at 37 °C for 24 h. Thereafter, astrocytes were exposed to 0.2 μmol/L STS (Biochrom, Germany) in the presence of 1 μg/mL SAL for other 24 h. Thereafter, the WST-1 assay was done to examine the cell viability. The experiments were repeated for five times independently.

2.5 Determination of intracellular ROS

Cellular ROS generation was determined by 2′,7′-dichlorohydro fluorescein diacetate (DCF-DA, Invitrogen, USA) assay using a microplate reader. For the assay, astrocytes were seeded in 96-well plate at a density of 3×10^4 cells per well. After preincubation with FCS-free medium for 6 h, astrocytes were treated with 1 μg/mL SAL for 24 h. After 24 h, astrocytes were incubated with 25 μmol/L DCF-DA dissolved in HBSS buffer in the absence or presence of 1 μg/mL SAL at 37 °C. Fluorescence was detected by excitation at 504 nm and emission at 530 nm using a microplate reader. Before reading fluorescence data, the plates were incubated for 10 min at 37 °C without light exposure.

2.6 Data analysis

All data are expressed as $\bar{x} \pm s$. One-way analysis of variance (ANOVA), followed by Dunnett's test was used to evaluate the data with comparison to control, or by Newman-Keuls test to assess the statistical significance between different groups. The comparisons between two groups were analyzed by Student's *t*-test. $P < 0.05$ was considered as significant.

3. Results

3.1 Structure identification of SAL

White needles (methanol), mp 158−159 °C, ^{1}H-NMR (500 MHz, in DMSO-d_6 with TMS as internal standard) δ: 2.71−2.74 (2H, m, Glc-H-6), 2.95, 3.03 (2H, 2m, H-2), 3.07, 3.12 (2H, 2m, H-1), 3.42−3.88 (4H, 4m, Glc-H-2, 3, 4, 5), 4.16 (1H, d, J = 7.8 Hz, Glc-H-1), 4.45 (1H, t, J = 5.9 Hz, Glc-6-OH), 4.85−4.89 (3H, 3s, Glc-2, 3, 4-OH), 6.65 (2H, d, J = 8.6 Hz, H-4, 8), 7.03 (2H, d, J = 8.6 Hz, H-5, 7), 9.12 (1H, s, 6-OH). ^{13}C-NMR (125MHz, in DMSO-d_6 with TMS an internal standard) δ: 70.7 (C-1), 35.5 (C-2), 129.3 (C-3), 130.4 (C-4, 8), 115.7 (C-5, 7), 156.3 (C-6), 103.5 (Glc-C-1), 74.1 (Glc-C-2), 77.4 (Glc-C-3), 70.8 (Glc-C-4), 77.5 (Glc-C-5), 61.8 (Glc-C-6).

3.2 Cell viability of astrocytes

In order to clarify if SAL had any beneficial effects on the cell viability, astrocytes were treated with SAL at the concentration of 1, 5, 10, 20, and 30 μg/mL for 48 h. As a result, compared with the untreated astrocytes, SAL significantly increased the viability of astrocytes at all the concentration indicated above. Cell viability in the group treated with SAL at 1, 5, 10, 20, and 30 μg/mL was (35 ± 11)%, (31 ± 9)%, (30 ± 9)%, (29 ± 9)%, and (18 ± 5)% above the control value, respectively (Figure 1). Interestingly, from the data of the cell viability assay, the lowest concentration of SAL (1 μg/mL) significantly increased cell viability to the maximum value, thus 1 μg/mL SAL was taken as the optimal concentration for our further experiments.

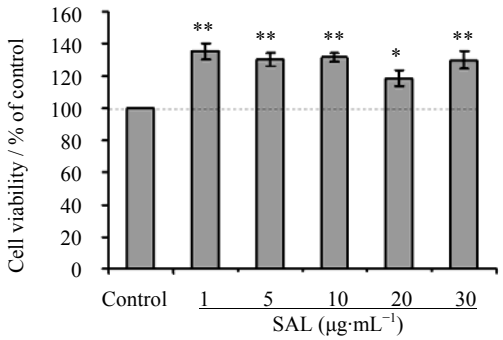

Figure 1 Concentration dependence of SAL to improve viability of astrocytes ($\bar{x} \pm s$)

$^*P < 0.05$ $^{**}P < 0.01$ *vs* control

3.3 Effects of SAL on cell viability of cortical neurons

Cortical neurons were treated with different concentration of SAL (1, 5, 10, 20, and 30 μg/mL) for 48 h. Thereafter, the WST-1 assay was carried out to evaluate the viability of neurons. Compared with the cortical neurons without treatment with SAL, neurons treated with 1, 5, 10, 20, and 30 μg/mL of SAL showed no significant differences in cell viability, indicating that SAL had no effects on improving cell viability of cortical neurons (Figure 2).

3.4 Protective effects of SAL against STS-induced cell death

SAL (1 μg/mL) was already confirmed to effectively increase the viability of astrocytes under FCS-free conditions.

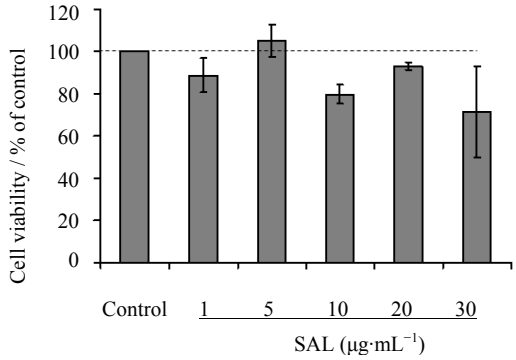

Figure 2 No effects of SAL on viability of hippocampus neurons ($\bar{x} \pm s$)

To further explore if the optimal concentration of SAL was capable of increasing cell viability in the presence of toxic stimuli, we firstly treated astrocytes with 1 μg/mL of SAL for 24 h. After that, astrocytes were treated with 0.2 μmol/L STS in the absence or presence of 1 μg/mL SAL for further 24 h. Cell viability under the different treatments was assessed by WST-1 assay. Compared with the untreated cells, astrocytes treated with STS showed 30% reduction in cell viability. However, compared with the astrocytes treated with STS, the combined treatment of STS and SAL caused a relatively higher percentage of cell viability (86% *vs* 70%). In conclusion, 1 μg/mL SAL could significantly increase cell viability with the STS stimulus, suggesting that SAL had the protective effects on astrocytes (Figure 3).

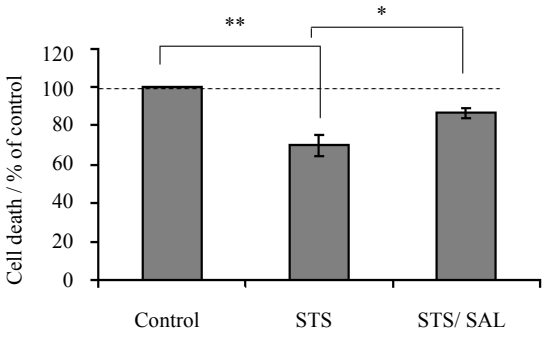

Figure 3 Protective effects of SAL on STS-induced cell death in astrocytes ($\bar{x} \pm s$)

$^{*}P < 0.05$ $^{**}P < 0.01$ *vs* control

3.5 Inhibition of SAL on spontaneous ROS production

One of the most outstanding characteristics is the anti-oxidative effect produced by SAL, which was already confirmed by the experimental data from multiple cell models, including human umbilical vein endothelial cells (Xu et al, 2013), endothelial (Leung et al, 2013), and hepatocytes (Yuan et al, 2013). Although SAL was reported to improve the impaired hippocampal neurogenesis in the rat model of Alzheimer's disease through scavenging intracellular ROS (Qu et al, 2012), it is still unclear if SAL is able to decrease the spontaneous intracellular oxidative reaction in astrocytes. To make it clear whether SAL has any effects on inhibiting oxidative effects in astrocytes, we used the DCF-DA assay to measure the spontaneous intracellular ROS production in the absence or presence of SAL in astrocytes. The ROS generation was monitored at the time points of 30 (A), 90 (B), and 120 min (C), respectively (Figure 4). With the treatment of 1 μg/mL SAL, the spontaneous intracellular ROS production in astrocytes was significantly inhibited at 90 and 120 min.

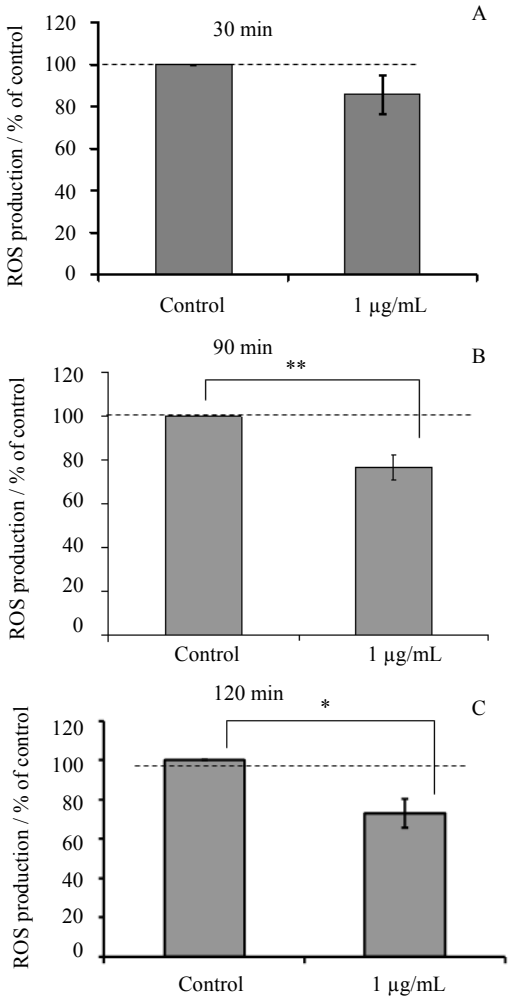

Figure 4 Decreases of SAL on intracellular spontaneous ROS generation in astrocytes ($\bar{x} \pm s$)

$^{*}P < 0.05$ $^{**}P < 0.01$ *vs* control

3.6 Inhibition of SAL on STS-induced ROS production in astrocytes

We demonstrated above in Figure 4 that 1 μg/mL SAL had anti-oxidative effects in astrocytes to attenuate the spontaneous intracellular ROS production. We were wondering if 1 μg/mL SAL would decrease STS-induced intracellular ROS release in astrocytes. To answer this question, astrocytes were incubated with or without 1 μg/mL SAL for 24 h. After that, astrocytes were treated with 0.2 μmol/L STS in the absence or presence of 1 μg/mL SAL. ROS was measured at the time points of 30, 90, and 120 min

by DCF-DA assay, as described above. According to these data, astrocytes treated with 0.2 μmol/L STS were found to have higher ROS level as compared with the untreated cells. However, with the application of SAL, the STS-induced ROS generation was significantly reduced at the time points of 30 (A) and 90 min (B) (Figure 5). Interestingly, this inhibition was not seen any more after 120 min (C). This manifested that SAL had the anti-oxidative effects in STS-treated astrocytes.

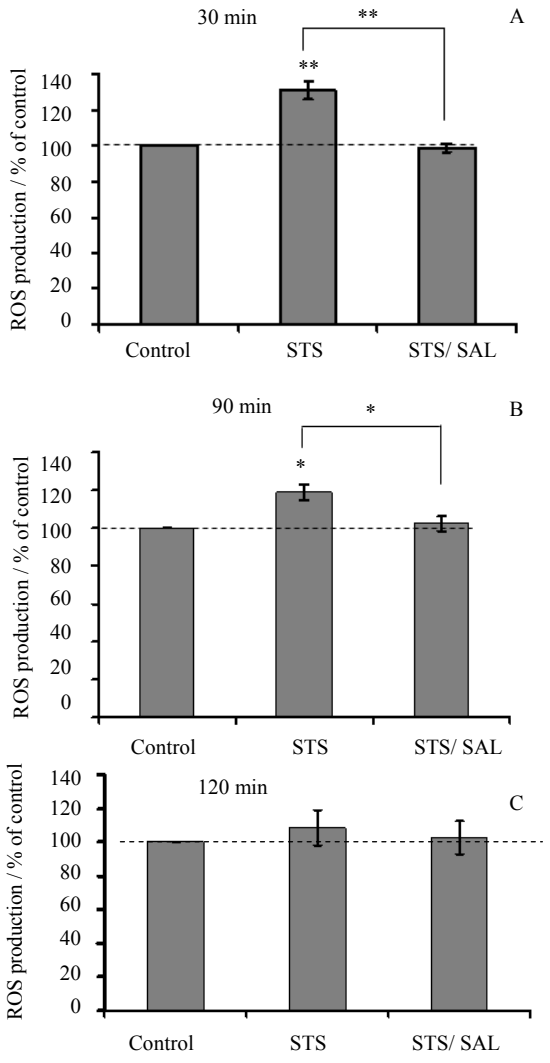

Figure 5 Attenuation of SAL on intracellular ROS generation induced by STS in astrocytes

$^{*}P < 0.05$ $^{**}P < 0.01$ *vs* control

4. Discussion

SAL is a major active ingredient isolated from *R. rosea*, which has been used as an adaptogen in traditional Tibetan medicine. SAL has a good reputation to possess a wide range of pharmaceutical properties, including the protective effects in the CNS. The neuroprotective effects exerted by SAL largely relied on its property of scavenging ROS (Qu et al, 2012). However, there were no reports whether there were beneficial effects from SAL on astrocytes. In the present study, we for the first time examined whether SAL was capable of improving the cell viability in the serum-deprived situation. Our results showed that the low concentration of SAL (1 μg/mL) successfully promoted the cell viability of astrocytes. The most interesting point was that with increasing the concentration, SAL failed to improve the cell viability any more (Figure 1). This is quite different from the results seen in other studies, confirming that SAL has the beneficial effects in a dose-dependent manner (Chen et al, 2008; 2009; Yu et al, 2008; Zhang et al, 2007). According to our data shown in Figure 1, the other concentration of SAL which is lower than 1 μg/mL or higher than 30 μg/mL may also produce the beneficial effects on astrocytes viability. This will be an interesting issue which is worth for future study. Although many studies revealed that SAL has the protective effects on different neurons or neuron-like models (Chen et al, 2008; Yu et al, 2008; Zhang et al, 2007), we failed to reproduce these effects in rat cortical neurons in our study (Figure 2).

STS as a bacterial alkaloid inhibits several cellular kinases (Ruegg and Burgess, 1989; Tamaoki et al, 1986). STS is frequently used as an inducer of cell death or apoptosis. The intracellular cascades regulated by STS have not been completely identified, but many studies have indicated that STS induced the release of cytochrome c, caspase activation (Krohn et al, 1998; 1999), and intracellular ROS accumulation (Krohn et al, 1998; Kruman et al, 1998; Prehn et al, 1997). In this study, we used STS-generated insults in astrocytes to partially model the pathological process in order to examine neuroprotective effects of SAL. Our result demonstrated that the exposure to STS caused a loss of cell viability of primary astrocytes, while treatment with SAL (1 μg/mL) attenuated the loss of cell viability induced by STS in astrocytes (Figure 3).

In our present study, we also found that STS induced ROS production in astrocytes (Figure 5). This could be one of the mechanisms accounting for the cell death induced by STS. To explore whether SAL has any inhibitory effects on ROS production in astrocytes, we first monitored the spontaneous ROS production in the absence or presence of SAL. According to these results, SAL was able to decrease the spontaneous ROS generation in astrocytes (Figures 4B and 4C). On the other hand, SAL significantly blocked STS-induced ROS release in astrocytes (Figure 5A and 5B). Consequently, SAL was demonstrated to have the anti-oxidative effects in astrocytes, which might be one of the important molecular mechanisms accounting for the cytoprotection. SAL was indicated to prevent aging in accelerated mouse aging model induced by *D*-galactose (Zhang et al, 2014). The newly found effect against spontaneous ROS production in this study may also make an important contribution to the mechanisms explaining the anti-aging effects of SAL, providing a new insight into the potential preventive value of SAL against neurological disease.

In summary, our results manifested that SAL increased cell viability of serum-deprived and STS-treated astrocytes. In addition, SAL was demonstrated to attenuate ROS production in the spontaneous and in the STS-treatment situation. Our results provide important clues for the molecular mechanisms to explain the beneficial effects of SAL to rescue astrocytes from STS-caused cell death.

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