

Salidroside protects against hydrogen peroxide-induced injury in HUVECs via the regulation of REDD1 and mTOR activation

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Abstract. Antioxidative therapy is considered an effective strategy for treating oxidative stress-induced apoptosis in cardiovascular diseases. Salidroside has been used as an antioxidative therapy for oxidative injury in cardiac diseases. However, the mechanism underlying its antioxidant effect is poorly understood. The present study aimed to investigate the pharmacological effects of salidroside on cultured human umbilical vein endothelial cells (HUVECs) under conditions of oxidative injury induced by hydrogen peroxide (H₂O₂) and the underlying mechanisms *in vitro*. HUVECs pretreated with or without salidroside for 24 h were exposed to H₂O₂-induced oxidative stress conditions for 6 h and then cell viability, apoptosis, HIF-1 α , regulated in development and DNA damage responses-1 (REDD1) and the PI3K/Akt/mTOR pathway were investigated. The results demonstrated that salidroside effectively attenuated H₂O₂-impaired cell viability and the production of reactive oxygen species (ROS) in a concentration-dependent manner. Reduced H₂O₂-induced apoptosis and activation of the cellular PI3K/Akt/mTOR pathway were demonstrated in HUVECs pretreated with salidroside. Furthermore, the level of REDD1, a direct regulator of mitochondrial metabolism, significantly increased in parallel with the level of HIF-1 α following pretreatment with salidroside. The antioxidative effect of salidroside was abrogated in REDD1 knockdown cells. However, LY294002, a PI3K inhibitor, attenuated the anti-apoptotic effect of salidroside and blocked the increase of Akt and mTOR; however, did not affect the antioxidative effect of salidroside. These findings suggested that salidroside was capable of protecting HUVECs against H₂O₂-induced

apoptosis by activating the PI3K/Akt/mTOR-dependent pathway and inhibiting ROS production by activating REDD1.

Introduction

There is evidence that oxidative stress is a major stimulus in the pathogenesis of cardiovascular diseases, including atherosclerosis, hypertension, myocardial infarction and heart failure (1,2). Hypoxia and ischemia may increase the production of intracellular reactive oxygen species (ROS) and consequently induce cardiomyocyte necrosis and apoptosis (3,4). Therefore, the development of an effective antioxidant strategy to reduce oxidative stress, which has attracted considerable interest from investigators, may provide insights into delaying or preventing cardiomyocyte death in ischemic heart diseases (5).

The mammalian target of rapamycin (mTOR) is the central controlling mechanism of cellular growth and metabolism. mTOR stimulates protein synthesis and cell growth through the inhibition of eukaryotic initiation factor 4E-binding protein-1 (4EBP1) and the activation of ribosomal protein S6 kinase (S6K) (6-8). The Akt-mediated activation of the mTOR pathway is a major regulator of the cellular response to hypoxia and other microenvironmental stresses (9). Several proteins are involved in the regulation of mTORC1 activity, including proline-rich Akt substrate of 40 kDa (PRAS40), DEP domain-containing mTOR-interacting protein (DEPTOR) or regulated in development and DNA damage responses-1 (REDD1) (10-12).

REDD1, as a hypoxia-regulated HIF-1 target gene, is important in the TSC1/TSC2-mediated inhibition of mTOR (13). REDD1 is markedly induced by numerous stress stimuli, including hypoxia, oxidative stress and energy depletion. More recent studies have suggested that REDD1 functions as a direct regulator of mitochondrial metabolism; however, the molecular mechanisms by which cell stress and hypoxia induce REDD1 expression have yet to be determined (14,15).

Salidroside (Fig. 1), as an adaptogen purified from *Rhodiola rosea* (*R. rosea*), has been reported to exert antioxidative, anti-aging, neuroprotective and cardioprotective effects, implying that salidroside may play a central role in the alleviation of mitochondrial-generated ROS and modulation of mitochondrial-related apoptosis signaling in multiple

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types of cells (16). A previous study has demonstrated that pretreatment with salidroside upregulated the HIF-1 α protein and induced its translocation in order to protect cardiomyocytes against hypoxia-induced injury (17). Nevertheless, the mechanism underlying its antioxidative effects remains unclear and requires further investigation.

In the present study, we investigated the potential anti-apoptosis activity of salidroside using human umbilical vein endothelial cells (HUVECs). Our results indicated that salidroside markedly inhibits hydrogen peroxide (H₂O₂)-induced HUVEC apoptosis and ROS production. Furthermore, we demonstrated that the anti-apoptosis effect of salidroside may be mediated by activation of the PI3K/Akt/mTOR pathway. Furthermore, the antioxidative effect of salidroside is dependent on REDD1 activity and requires the transcription factor HIF-1 α .

In conclusion, our results demonstrated that the activation of REDD1 by salidroside affects cell viability and ROS production, and may be a promising candidate for antioxidant therapy in cardiovascular disease.

Materials and methods

Materials. Salidroside, an active component of the medicinal plant *R. rosea*, with 99% purity confirmed by HPLC, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) and Lipofectamine™ 2000 were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA); FITC-conjugated annexin-V was purchased from BD Biosciences (San Jose, CA, USA); and LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and treatment. HUVECs (ATCC, Teddington, UK) were cultured in F-12K medium with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 30 μ g/ml endothelial cell growth supplement and 10% FBS at 37°C in 5% CO₂ and 95% humidity. For H₂O₂ treatment, cells were first screened with increasing doses of H₂O₂ and the optimal concentration (200 μ mol) was selected for further experiments. For antioxidant treatment, the optimal concentration (100 μ g/ml) of salidroside was selected for further experiments. Cells were cultured with salidroside or a saline vehicle for 24 h, followed by H₂O₂ (200 μ mol) treatment. For LY294002 treatment, cells were cultured with salidroside followed by LY294002 and H₂O₂ treatment.

Plasmid construction, lentiviral packaging and infection. To silence REDD1 expression, we constructed a vector-based REDD1-shRNA to interfere with the expression of REDD1. The oligonucleotides used were 5'-CCGGTGATGCCTAGCCAGTTGGTAACTCGAGTTACCAACTGGCTAGGCATCATTTTTG-3' and 5'-AATTCAAAAATGATGCCTAGCCAGTTGGTAACTCGAGTTACCAACTGGCTAGGCATCA-3', and were cloned into the AgeI and EcoRI sites of the pLKO.1-puro vector (Sigma Aldrich). Lentiviruses were created by co-transfecting HEK 293T cells with a packaging vector. Lentiviral supernatants were collected 48 h post-transfection and target cells were infected with 3 ml virus supernatant containing 8 μ g/ml polybrene for 24 h. The media was

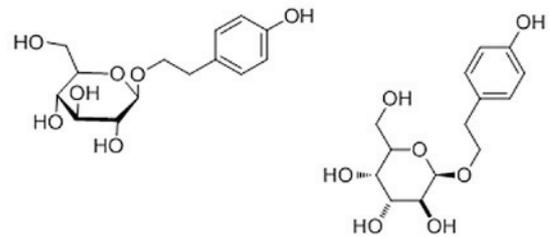


Figure 1. Chemical structure of salidroside (p-hydroxyphenethyl- β -D-glucoside).

replaced with complete medium. Puromycin (3 μ g/ml) was added to the cell culture media two days after infection. The knockdown efficiency was monitored by western blot analysis. The formation of REDD1-shRNA was confirmed using DNA sequencing.

Cell viability assay. Cells were seeded in 96-well culture plates at 10⁴ cells/well and allowed to attach to the plates. The culture medium was removed and renewed with fresh medium containing H₂O₂ at various concentrations, ranging from 0-400 μ M and incubated for 6 h. For the salidroside assays, cells were pretreated with salidroside for 24 h prior to the addition of H₂O₂. Cells receiving saline served as a vehicle control and were equivalent to no treatment. The Cell Counting kit-8 (CCK-8) reagent (Sigma Aldrich) was added and incubated at 37°C for 2 h according to the manufacturer's instructions. Optical density (OD) values at 450 nm were read by a microplate reader. Each experiment was performed three times independently.

Flow cytometric assays. To evaluate apoptosis in HUVECs, flow cytometric analysis was performed using annexin-V. Cells were treated with 100 μ g/ml salidroside or saline for 24 h, then 200 μ mol H₂O₂ was added. Cells were detached by exposure to trypsin 6 h later. Apoptosis was detected in cells washed with PBS by staining with annexin-V and propidium iodide according to the manufacturer's instructions (BD Biosciences). Stained cells were analyzed using flow cytometry with CellQuest (Becton-Dickinson, Franklin Lakes, NJ, USA) software.

The cells, which had been treated or remained untreated with salidroside or H₂O₂, were incubated with a chloromethyl derivative of H₂DCFDA (CM-H₂DCFDA; Invitrogen Life Technologies) in the dark for 30 min at 37°C. Following washing, the ROS level of cells was immediately analyzed by flow cytometry.

Protein extraction and western blot analysis. Cells were washed three times with ice-cold PBS and subsequently lysed in RIPA buffer containing 1 mM PMSF. The supernatant was collected and the protein concentration of each sample was determined using a BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions. Total proteins (50 μ g) were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and transferred onto 0.22- μ m PVDF membranes (Millipore, Billerica, MA, USA), which were blocked with 8% non-fat milk. The primary antibodies used to probe the membranes included

anti-HIF-1 α [Cell Signaling Technology, Inc. (CST), Beverly, MA, USA; #3716], anti-REDD1 (ProteinTech Group, Chicago IL, USA; 10638-1-AP), anti-Akt (CST, #9272), anti-p-Akt (CST, #9271), anti-p-p70S6K (CST, #6198), anti-p-mTOR (CST, #2971) and anti-GAPDH (ProteinTech Group, 60004-1-Ig) at 4°C overnight. Following washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Proteins were detected using an advanced enhanced chemiluminescence (ECL) system (GE Healthcare, Chalfont St. Giles, UK).

Statistical analysis. The results of all experiments are expressed as the means \pm SD from individual experiments. Data were compared using the Student's t-test using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Salidroside attenuates H_2O_2 -induced HUVEC cytotoxicity and excessive generation of ROS. In order to investigate the effect of salidroside on the growth of endothelial cells, we evaluated the effect of salidroside on the viability of HUVECs using the CCK-8 assay. H_2O_2 (50-400 μ mol) significantly decreased the cell viability of HUVECs in a concentration-dependent manner ($P < 0.01$; Fig. 2A). When exposed to 200 μ mol H_2O_2 for 6 h, HUVEC viability ($61.9 \pm 4.1\%$, $n=3$) decreased by 38.1% compared with the control group ($P < 0.01$, both $n=3$). Therefore, 200 μ mol of H_2O_2 was used as the optimal concentration in the subsequent experiments. To examine the concentration-dependent effect of salidroside, cells were pretreated with 20, 50 or 100 μ g/ml salidroside or saline vehicle for 24 h prior to being exposed to 200 μ mol H_2O_2 for 6 h. The decrease in HUVEC viability induced by H_2O_2 was improved significantly by treatment with salidroside in a concentration-dependent manner. A significant increase in cell viability was evident following pretreatment with 100 μ g/ml of salidroside (Fig. 2B). The optimal concentration (100 μ g/ml) of salidroside was selected for further experiments. In addition, salidroside at each concentration applied alone did not cause any apparent cytotoxicity (data not shown). The dichlorofluorescein (DCFH) assay results indicated that salidroside reduced the levels of ROS in HUVECs under H_2O_2 . When HUVECs were subjected to H_2O_2 for 6 h, the concentration of intracellular ROS increased, compared with the control group ($P < 0.01$; Fig. 2C). However, preincubation with 100 μ g/ml salidroside for 24 h followed by coincubation with 200 μ mol H_2O_2 for a further 6 h markedly decreased the H_2O_2 -induced ROS level. ROS production decreased by 22.9% compared with cells treated with H_2O_2 alone ($P < 0.01$; Fig. 2D).

Salidroside inhibits H_2O_2 -induced HUVEC apoptosis. CCK-8 does not discriminate between necrosis and apoptosis. We evaluated the possible anti-apoptotic effect of salidroside on HUVECs subjected to H_2O_2 using annexin-V/PI double staining and flow cytometry analysis. In the control group, the majority of cells were viable (93.7%). H_2O_2 increased the percentage of apoptotic cells. Compared with the control group, 6 h stimulation of H_2O_2 -induced apoptosis produced a

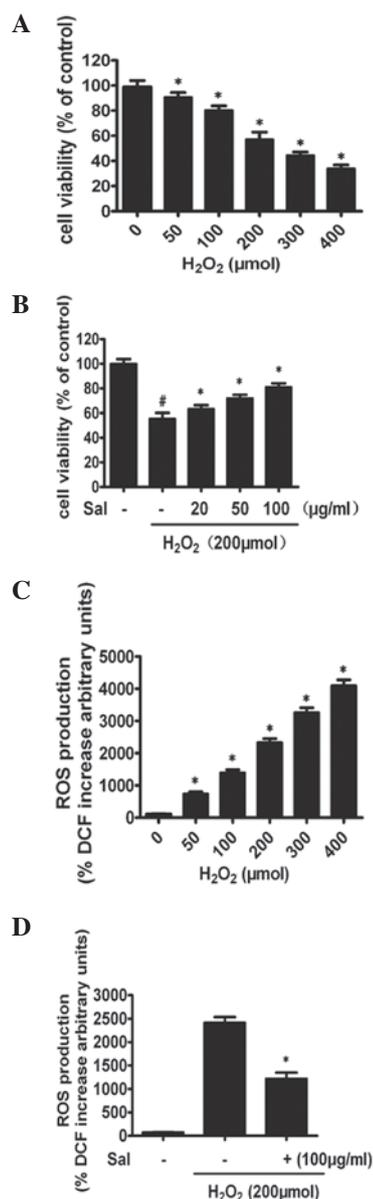


Figure 2. Sal attenuated H_2O_2 -induced HUVEC cytotoxicity and excessive generation of ROS. (A) Concentration-dependent toxic effect of H_2O_2 on the viability of HUVECs. (B) Effect of Sal on H_2O_2 -induced HUVEC death. (C) ROS contents in HUVECs under H_2O_2 stimulation. (D) Changes in ROS induced by H_2O_2 alone or in combination in the absence or presence of Sal, respectively. All data are presented as the means \pm SEM of the three experiments and each included triplicate sets. * $P < 0.01$ vs. control group; # $P < 0.01$ vs. 100 μ g/ml Sal group. Sal, salidroside; HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species.

5.3-fold increase in apoptotic cells ($6.5 \pm 0.8\%$, $P < 0.01$, $n=3$; Fig. 3A and B). Pretreatment with salidroside for 24 h and cotreatment with H_2O_2 for a further 6 h significantly attenuated the apoptosis induced by H_2O_2 , with 51.5% fewer apoptotic cells compared with the cells treated with H_2O_2 alone ($P < 0.01$, $n=4$; Fig. 3C and D).

Salidroside increases the expression of REDD1 protein and activates the Akt/mTOR pathway. HIF-1 α reduces the production of ROS under hypoxic conditions, which mediate the cells adaptive response to hypoxia and ischemic. It is doubtful as to whether REDD1 is also associated with the process of

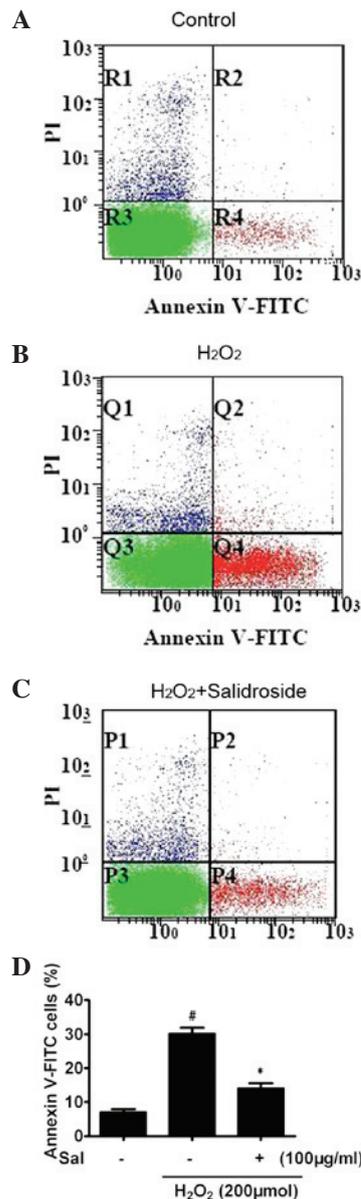


Figure 3. Sal inhibited HUVEC apoptosis induced by H₂O₂ stimulation. (A-C) Representative graphs of cells with various treatments are displayed. Number of annexin V⁺/PI⁺-stained cells decreased significantly in the Sal treatment groups. (D) Sal treatment attenuated cell death and late apoptosis in HUVECs induced by H₂O₂. [#]P<0.01, compared with the control group. ^{*}P<0.01, compared with the H₂O₂ group. HUVECs, human umbilical vein endothelial cells; Sal, salidroside.

salidroside protection against H₂O₂-induced ROS. Therefore, we investigated the levels of REDD1 and consequently, the expression of HIF-1 α in response to H₂O₂ and salidroside. HUVECs were stimulated with salidroside for 24 h and subsequently with H₂O₂ for a further 6 h; following this, the REDD1 and HIF-1 α protein levels were analyzed. Incubation of HUVECs in H₂O₂ stimulated REDD1 expression (Fig. 4A). Notably, this expression was enhanced when the HUVECs were incubated with salidroside under H₂O₂. This result indicates that REDD1 may be activated under H₂O₂ conditions and salidroside. Similarly, H₂O₂ and salidroside conditions led to an increase in the levels of HIF-1 α protein, consistent with previous findings.

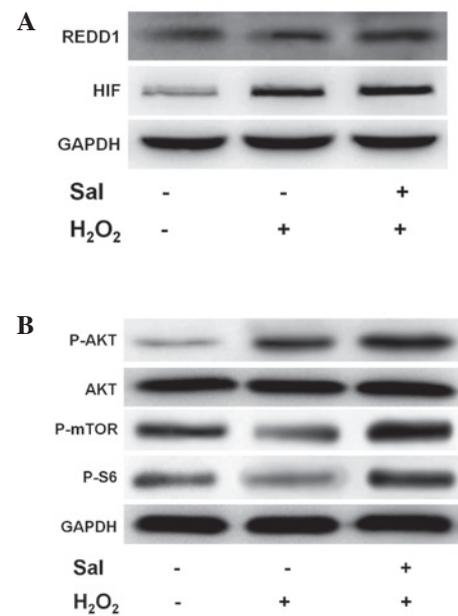


Figure 4. Sal increased the expression of REDD1 protein and activated the Akt/mTOR pathway. (A) Sal increased the expression of REDD1 protein in HUVECs under H₂O₂ stimulation. (B) Sal activated the AKT/mTOR pathway in HUVECs under H₂O₂ stimulation. Total Akt and GAPDH expression are shown as sample loading controls. The immunoblot is representative of n=3 experiments. REDD1, regulated in development and DNA damage responses-1; HUVECs, human umbilical vein endothelial cells; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Sal, salidroside.

In order to fully elucidate the potential signaling pathway involved in anti-apoptotic induction by salidroside under oxidative stress conditions in HUVECs, we examined the implication of protein kinases activated in response to salidroside. The mTOR pathway has been reported to enhance translation and transcription, enabling cell growth. Therefore, we investigated whether salidroside activated mTOR in the HUVECs treated with H₂O₂, and if so, whether Akt modulates this activation. Therefore, HUVECs were pretreated with salidroside and subjected to 6 h of treatment with H₂O₂. We performed western blot analysis in order to detect the effect of salidroside on the phosphorylation of Akt and mTOR in HUVECs. We observed that H₂O₂ and salidroside pretreatment increased the expression of phospho-Akt (serine 473; Fig. 4B). However, mTOR and S6 phosphorylation decreased significantly in HUVECs treated with H₂O₂ alone. Notably, phosphorylation levels were significantly elevated following pretreatment with salidroside (Fig. 4B).

Antioxidative effect of salidroside is dependent on REDD1 activation. To determine whether salidroside-induced antioxidatives require the function of REDD1, we examined the effect of transfecting HUVECs with siRNAs targeting REDD1. Initially, we examined the effect of siRNAs targeting REDD1. Western blot analysis revealed that siRNAs were highly effective at decreasing REDD1 protein levels (Fig. 5A). Furthermore, REDD1 siRNAs significantly reduced the salidroside-induced antioxidative effect (Fig. 5B). Thus, salidroside protects against oxidative stress-induced cell death and this protective effect is dependent upon REDD1 activation.

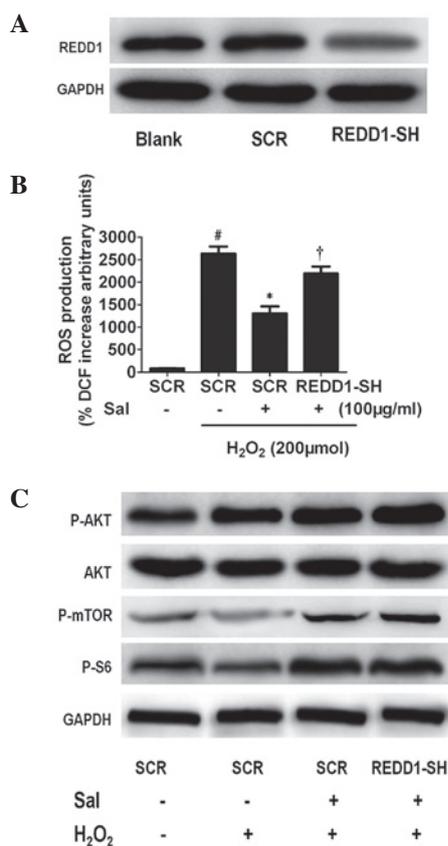


Figure 5. The antioxidative effect of Sal is dependent on REDD1 activation. (A) Knockdown of REDD1 in HUVECs by lentiviral REDD1-directed shRNA (shREDD1) or the control group, assessed by western blot analysis. GAPDH served as a loading control. (B) REDD1 knockdown decreases the antioxidative effect of Sal under H₂O₂. Equal numbers of the indicated REDD1^{-/-} HUVECs were treated under H₂O₂ with or without Sal, and increased ROS production in whole REDD1^{-/-} versus wild-type cells was measured using DCFH. [#]P<0.01, compared with the control. ^{*}P<0.01, compared with the H₂O₂ treatment group. [†]P<0.01, compared with the SCR group. (C) Western blot analysis shows that Akt/mTOR pathway expression remained upregulated in REDD1-depleted HUVECs as well as untransfected and control siRNA HUVECs subjected to hypoxic stress. REDD1, regulated in development and DNA damage responses-1; HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species; GAPDH, glyceraldehyde 3-phosphate dehydrogenase, DCFH, dichlorofluorescein; Sal, salidroside.

These data also confirm that activation of REDD1 is protective against oxidative stress.

PI3K participates in the regulation of mTOR expression by salidroside. To determine whether PI3K activation is involved in the induction of mTOR expression by salidroside, we treated HUVECs with the PI3K inhibitor LY294002. We subjected HUVECs to salidroside for 24 h under H₂O₂ conditions in the absence or presence of inhibitors, and mTOR expression was detected by immunoblotting. Salidroside and H₂O₂ stimulate Akt expression, and the combination of the treatments led to an additive effect. However, LY294002 blocked the effect of salidroside on the expression of mTOR. Furthermore, LY294002 did not prevent the effect of ROS production (Fig. 6). These results suggested that in HUVECs, salidroside stimulates mTOR expression through PI3K/mTOR signaling pathways. These data demonstrate that Akt may be a central regulator of the mTOR pathway for the anti-apoptotic effect of salidroside.

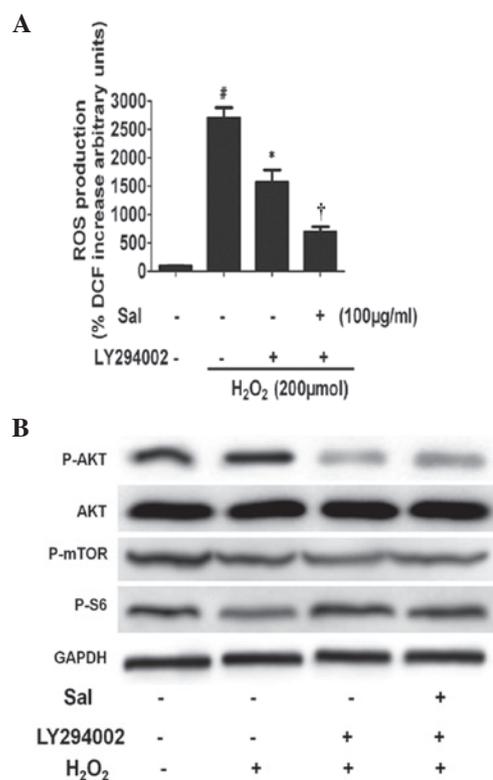


Figure 6. The PI3K pathway participates in the regulation of mTOR expression by Sal. (A) Changes in ROS production induced by either Sal or H₂O₂ treatment alone or in combination in the absence and presence of LY294002, respectively. HUVECs were preincubated with Sal, and then coincubated with H₂O₂ for a further 6 h, and LY294002 was added 30 min prior to treatment with Sal. [#]P<0.01, compared with the control. ^{*}P<0.01, compared with the H₂O₂ treatment group. [†]P<0.01, compared with the H₂O₂ + LY294002 treatment group. (B) Activation of the mTOR pathway by Sal is dependent on PI3K/Akt. PI3K/Akt acted in parallel to activate the mTOR pathway following treatment with Sal. The PI3K inhibitor LY294002 blocks the increase in mTOR and PS6 protein levels induced by Sal under H₂O₂ conditions. Levels of phosphorylated Akt (Ser473), mTOR and p-p70S6K were determined by immunoblotting. Total Akt and GAPDH expression are shown as sample loading controls. A representative experiment of three independent experiments is shown. HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Sal, salidroside.

Discussion

In the present study, we demonstrated that increased REDD1 expression in response to salidroside may participate in the mechanism by which salidroside inhibited H₂O₂-induced HUVEC oxidative stress, and salidroside-induced mTOR expression occurs through the PI3K/mTOR signaling pathway to protect HUVECs against apoptosis induced by H₂O₂.

The necrotic and apoptotic processes induced by oxidative stress are involved in the pathogenesis of cardiac ischemic-reperfusion injury, which may ultimately lead to heart failure (1,18). Our present study confirmed that H₂O₂ significantly decreases cell viability, as evidenced by CCK-8, whereas pretreatment with various concentrations (10, 50 and 100 μg/ml) of salidroside attenuated the H₂O₂-decreased cell viability in a dose-dependent manner. These results indicated that salidroside exhibited an apparent protective effect against

H₂O₂-induced cytotoxicity and inhibited ROS production in response to H₂O₂ in HUVECs.

Recently, there has been increased interest in the role of the mTOR pathway in the heart. While the evidence is mounting that the activation of mTOR may be an important cellular signaling event for preventing apoptotic processes, the role of mTOR in regulating cardioprotection is less clear in that it has been examined in a number of settings with what appear to be disparate conclusions (19,20). Recent studies have examined the role of Akt in the protective effect of salidroside, and the conclusions reached were that salidroside prevented apoptosis in neuronal and cardiac cells, through the activation of the PI3K/Akt pathway (21,22). These findings indicated the regulation of Akt by salidroside, which aroused our interest in the role of mTOR and the downstream target, p70S6K, in the heart. In addition, examination of the regulation of salidroside may be crucial in identifying the molecular mechanisms that regulate salidroside in the heart under conditions of oxidative stress. Thus, we aimed to further characterize the regulation of mTOR by salidroside.

Therefore, we determined whether salidroside affected the phosphorylation of the mTOR pathway in HUVECs. In this study, we identified one key anti-apoptotic role for salidroside under H₂O₂: The activation of mTOR. The activation of mTOR pathway members was analyzed by western blot analysis using the specific antibody against the phosphorylated form of mTOR and SP6. We observed that treatment of HUVECs with salidroside increased the level of mTOR phosphorylation and SP6.

Previous studies have reported that salidroside induced the anti-apoptotic effect, so we also examined the cell apoptosis of HUVECs exposed to salidroside. As shown in Fig. 3, following the treatment of the cells with an increased concentration of salidroside for 24 h, a significant decrease in apoptosis occurred in HUVECs, which may be correlated to the salidroside-induced upregulation of mTOR.

We demonstrated that oxidative stress inactivates mTOR. Numerous studies have demonstrated that this inactivation is dependent on HIF activity and HIF-inducible REDD1 (12,13,23,24). REDD1, an important HIF-1 effector, is important in the TSC1 (hamartin)/TSC2 (tuberin)-mediated inhibition of mTOR (13). However, Hernández *et al* discovered that REDD-1 is downregulated by ROS by inactivating TSC2 and activating mTOR (25). However, our results indicate that stimulation with H₂O₂ increased REDD1 expression, which coincided with the upregulated expression of HIF-1 α in HUVECs. Furthermore, we observed that pretreatment with salidroside followed by coincubation with H₂O₂, increased REDD1 and HIF-1 α levels compared with those treated by H₂O₂ alone. The synergy between salidroside and H₂O₂ is due to the fact that they regulate REDD1 through distinct mechanisms. Although our data support a regulatory role for REDD1 under oxidative stress conditions, we observed the upregulation of mTOR following treatment with salidroside, which varies from the mTOR inhibition of oxidative stress occurring independently of REDD1. We propose that mTOR regulation involves multiple pathways, including mediation by the AMPK/TSC2/Rheb pathway and inhibition by the HIF/REDD1/2 pathway or the other HIF-independent pathways affected by mTOR, including PI3K/Akt. In this regard,

the elucidation of the basic mechanisms affecting this network of signaling molecules will offer essential information to improve our understanding of salidroside as well as supporting the pharmacological mechanism-based application of salidroside.

Akt is an important survival kinase in cell proliferation and survival, which is activated downstream of PI3K (26). Akt-mediated protection inhibits cardiomyocyte necrotic and apoptotic death induced by deleterious stimuli (27). The existence of several possible routes by which Akt modulates the mTOR pathway suggests that this may be a central mechanism of mTOR regulation. This study aimed to identify whether PI3K signaling pathways regulated mTOR activity in HUVECs exposed to salidroside under oxidative stress conditions. Herein, we delineate a novel signaling pathway regulated by salidroside-activated Akt that leads to the activation of mTOR. This conclusion is supported by the following results: i) Under oxidative stress conditions, salidroside induces markedly higher phosphorylation levels of Akt compared with those treated by H₂O₂ alone, which is similar to the result reported by Zhu *et al* (22); ii) LY294002 efficiently suppressed the activation of Akt and the induction of mTOR expression by salidroside under oxidative stress conditions; iii) LY294002 inhibited the phosphorylation of mTOR; iv) the downregulation of mTOR inhibited the activation of SP6; and v) mTOR expression was unaffected by siRNAs targeting REDD1. We delineated the pathway upstream of mTOR activation in response to salidroside under oxidative stress, defining Akt as an essential activator of the mTOR pathway in this situation.

Due to the fact that REDD1 is essential in limiting the generation of ROS by an unidentified mechanism and in ROS attenuation by salidroside, we hypothesized that the mechanism by which salidroside inhibits H₂O₂-induced oxidative stress in HUVECs lies in the upregulation of the REDD1 protein. To more clearly define the role of REDD1, we examined the production of ROS in REDD1-knockdown HUVECs. The REDD1^{-/-} cells demonstrated a substantial elevation in ROS production, and treatment with salidroside was insufficient to normalize the ROS levels to wild-type levels (Fig. 5).

In conclusion, we demonstrated that salidroside protects HUVECs against oxidative injury induced by H₂O₂ and that the potential mechanisms may involve increasing REDD1 expression to prevent the generation of ROS, modulating the expression of HIF-1 α and regulating the activation of the PI3K/Akt pathway followed by activating the downstream molecules of mTOR and SP6 to reduce H₂O₂-induced apoptosis. These combined effects may explain the marked protective effect of salidroside.

Acknowledgements

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