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Salidroside inhibits oxygen glucose deprivation (OGD)/re-oxygenation-induced H9c2 cell necrosis through activating of Akt–Nrf2 signaling

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ABSTRACT

Oxygen glucose deprivation (OGD)/re-oxygenation has been applied to cultured cardiomyocytes to create a cellular model of ischemic heart damage. In the current study, we explored the potential role of salidroside against OGD/re-oxygenation-induced damage in H9c2 cardiomyocytes, and studied the underlying mechanisms. We found that OGD/re-oxygenation primarily induced necrosis in H9c2 cells, which was inhibited by salidroside. Salidroside suppressed OGD/re-oxygenation-induced reactive oxygen species (ROS) production, p53 mitochondrial translocation and cyclophilin D (Cyp-D) association as well as mitochondrial membrane potential (MMP) decrease in H9c2 cells. Meanwhile, salidroside activated Akt and promoted transcription of NF-E2-related factor 2 (Nrf2)-regulated genes (heme oxygenase-1 (HO-1) and quinone oxidoreductase 1 (NQO-1)). Significantly, Nrf2 shRNA knockdown or Akt inhibitors (LY 294002 and wortmannin) not only prevented salidroside-induced HO-1/NQO-1 transcription, but also alleviated salidroside-mediated cytoprotective effect against OGD/re-oxygenation in H9c2 cells. These observations suggest that salidroside activates Nrf2-regulated anti-oxidant signaling, and protects against OGD/re-oxygenation-induced H9c2 cell necrosis via activation of Akt signaling.

1. Introduction

Ischemic heart disease and many cardiovascular diseases (CVD) are major threat to human health and important contributors of human mortality around the world. Thus, its pathogenesis and possible prevention strategies are important [1,2]. Recently, many new technologies for interventional treatment have resulted in rapid increases in the treatment efficiency of CVD, and quality of life has been greatly improved for many patients [3,4]. However, conservative drug treatment is still recognized as an effective and safe method for enhancing both the length and quality of life. Oxygen glucose deprivation (OGD) has been applied in cultured cardiomyocytes to create a cellular model of ischemic heart damage [5–7]. Serve and/or sustained OGD (>1 h) disrupts cell mitochondrial functions by inhibiting mitochondrial complex-I activity, and when coupling with re-oxygenation, superoxide and other reactive oxygen species (ROS) will be produced, which cause oxidative stress and cell necrosis [5–7].

Salidroside (*p*-hydroxyphenethyl-β-D-glucoside) is the main active ingredient from *Rhodiola rosea* L, and has displayed many pharmacological properties including anti-aging, anti-fatigue, anti-oxidant, anti-cancer and anti-inflammation effects [8–12]. For example, salidroside inhibits hydrogen peroxide (H_2O_2)-induced damage of SH-SY5Y human neuroblastoma cells and rat hippocampal neurons [10,11]. Meanwhile, salidroside protects cells from glutamate toxicity, as well as calcium-overload and cobalt chloride stresses [9,12]. However, the potential role of salidroside against OGD/re-oxygenation-induced cardiomyocyte cell death is not fully-studied. More significantly, the underlying mechanism of salidroside-mediated cytoprotective effect is still largely unknown.

In the current study, we explored the potential role of salidroside against OGD/re-oxygenation in cultured H9c2 cardiomyocytes, and found that salidroside protected H9c2 cells from OGD/re-oxygenation through activating Akt dependent NF-E2-related factor 2 (Nrf2) signaling.

2. Material and methods

2.1. Chemical and reagents

Salidroside, doxorubicin and N-acetyl cysteine (NAC) were purchased from Sigma Chemical Company (St. Louis, MO).

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Z-DEVD-fmk, Z-VAD-fmk and necrostatin-1 were purchase from Calbiochem (Darmstadt, Germany). Antibodies against cyclophilin D (Cyp-D), Nrf2 and p53 were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Antibodies for Akt, phosphorylated (p-) Akt and β-actin were purchased from Cellular Signaling Tech (Beverly, MA).

2.2. H9c2 cell culture

As reported [13,14], rat embryonic ventricular H9c2 cardiomyocytes (a gift from Jiang Lai's group [14]) were maintained in DMEM medium, supplemented with a 10% FBS, penicillin/streptomycin, and 4 mM L-glutamine, in a CO_2 incubator at 37 °C.

2.3. H9c2 OGD/re-oxygenation model

H9c2 cells were placed in an anaerobic chamber (HERA cell 150, partial oxygen pressure was maintained below 2 mmHg). The medium was replaced with a pre-warmed (37 °C) glucose-free balanced salt solution (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 0.025 mM phenol red, and 20 mM sucrose). The solution was bubbled with an anaerobic gas mix (95% N_2, 5% CO_2) for 30 min. Cell cultures subjected to OGD were incubated in the solution at 37 °C for a 1–4 h to produce oxygen deprivation and then re-oxygenated (returned to the normal aerobic environment). Experimental parameters were assayed at 3–24 h following re-oxygenation.

2.4. Cell viability assay (MTT assay)

H9c2 cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) method as described [14]. The MTT cell viability OD value was detected.

2.5. Flow cytometry assay of cell death and apoptosis

After treatment, H9c2 cells were detached, washed and incubated in 500 μl binding buffer, 5 μl annexin V-FITC and 5 μl of propidium iodide (PI) (Invitrogen, Shanghai, China) at room temperature for 15 min in the dark. Cells were then detected through fluorescence-activated cell sorting (FACS) with a Becton-Dickinson FACScan (Immunocytochemistry Systems, San Jose, CA). Annexin V positive cells were labeled as apoptotic cells, and Annexin V negative but PI positive cells were marked as necrotic cells. PI percentage was utilized as cell death percentage.

2.6. Caspase-3 activity assay

After treatment, cytosolic proteins from approximately 1×10^6 H9c2 cells were extracted in hypotonic cell lysis buffer (25 mm HEPES, pH 7.5, 5 mm $MgCl_2$, 5 mm EDTA, 5 mm dithiothreitol, 0.05% phenylmethylsulfonyl fluoride). Protein concentration of samples was determined by a Bio-Rad Bradford protein assay kit (Bio-Rad, Shanghai, China). Twenty μg of cytosolic extracts were added to caspase assay buffer (312.5 mm HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS) with benzyloxycarbonyl-DEVD-7-amido-4-(trifluoromethyl) coumarin as the substrate (Calbiochem, Darmstadt, Germany). Release of 7-amido-4-(trifluoromethyl)coumarin (AFC) was quantified, after 2 h of incubation, using a Fluoroskan system (Thermo-Labsystems, Helsinki, Finland) set to an excitation value of 355 nm and emission value of 525 nm. The results were expressed as relative fluorescence units/μg of protein.

2.7. Western blots

Western blots were performed as described [14]. Prior to blotting, an SDS–PAGE gel was stained with Coomassie Blue to provide visual confirmation that equivalent amounts of the various samples were being analyzed. After the electrophoretically resolved proteins were blotted onto Immobilon-P, the membranes were stained with Ponceau S to verify that transfer of all samples was satisfactory. Band intensity was quantified and normalized to loading control.

2.8. Mitochondrial immunoprecipitation (Mito-IP)

Mitochondria of $1 * 10^7$ H9c2 cells with/out treatment were isolated using "Mitochondria Isolation Kit for Cultured Cells" from Thermo Scientific (Hudson, NH). The mitochondria were then lysed with lysis buffer (20 mM Tris, pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol and 1% Triton X-100). Immunoprecipitation (IP) was performed using anti-Cyp-D (see [15]), and immune complexes were captured with protein G-Sepharose. Proteins were resolved by SDS–PAGE, p53-Cyp-D association was detected by the Western blots.

2.9. Real-time PCR

Total RNA of H9c2 cells was extracted through TRIzol reagents (Invitrogen, USA), and reverse transcription was performed using TOYOBO ReverTra Ace-a RT-PCR kit (TOYOBO, Japan) according to the manufacturer's instructions. Real-time PCR was performed on a Bio-Rad IQ5 multicolor detection system based on suggestions from the supplier. One RNA sample of each preparation was processed without real time-reaction to provide a negative control in subsequent PCR. After amplification, melt curve analysis was performed to analyze product melting temperature. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was chosen as the reference gene for normalization, and the $2^{-\Delta\Delta CT\ (Cycle\ Threshold)}$ method [16] was applied to quantify targeted mRNA fold changes within samples. The primer set for rat HO-1 were: sense 5′-CAC GCA TAT ACC CGC TAC CT-3′, antisense 5′-AAG GCG GTC TTA GCC TCT TC-3′ [17]; The primer set for rat GAPDH were: sense 5′-CCA TCA CCA TCT TCC AGG AG-3′, antisense 5′-CCT GCT TCA CCA CCT TCT TG-3′ [17]; The primer set for rat NQO-1 were: sense 5′-GCAGGATTCGCCTACACGTATG-3′, antisense 5′-GGTGATGGAAAG CAAGGTCTTC-3′ [18].

2.10. ROS detection

ROS in H9c2 cells was determined by carboxy-H2DCFDA staining method. This assay is based on the principle that the non-polar, nonionic H2-DCFDA crosses cell membranes and is enzymatically hydrolyzed into non-fluorescent H2-DCF by intracellular esterase. In the presence of ROS, H_2-DCF is rapidly oxidized to become highly fluorescent DCF. Briefly, H9c2 cells were incubated at 37 °C for 30 min with 5 μM carboxy-H2-DCFDA dissolved in the culture medium. $5 * 10^5$ cells were resuspended in PBS, and sent to flow cytometry analysis (Epics Altra, BECKMAN, CA). The percent of fluorescence-positive cells as a measure of ROS generation was recorded on a spectrophotometer using excitation and emission filters of 488 and 530 nm, respectively.

2.11. Detection of mitochondrial membrane potential (MMP)

MMP was measured through JC-10 dye (Invitrogen, Carlsbad, CA) [19]. The JC-10 dye exhibits two staining spectra. In normally resting cells, the dye forms aggregates in the mitochondrial membrane, exhibiting orange fluorescence. When the membrane

potential is decreasing, the monomeric JC-10 will form in the cytosol, exhibiting the green fluorescence. Thus, the intensity of green fluorescence was detected as indicator of MMP loss. Briefly, H9c2 cells were stained with 5.0 μg/ml of JC-10 for 5 min at room temperature under dark. Cells were then washed twice with warm PBS, and resuspended in fresh culture medium and read immediately on a microplate reader with an excitation filter of 485 nm.

2.12. Nrf2 knockdown by shRNA

Two sets of lentiviral particles containing Nrf2 shRNAs (non-overlapping sequences) were designed and synthesized by Shanghai Kaiji Biotech (Shanghai, China). Lentiviral particles (20 μl/ml) were added to cells for 24 h, and cells expressing shRNA-containing lentivirus were selected by puromycin (0.25 μg/ml) for additional 48 h. Nrf2 and loading control (Akt) expression in selected cells was tested by Western blots. Control cells were transfected with same amount of scramble shRNA.

2.13. Statistical analysis

Statistical significance was determined by one-way ANOVA by Dunnett's test.

3. Results

3.1. Salidroside inhibits OGD/re-oxygenation-induced H9c2 cell necrosis

We first tested the potential role of salidroside against OGD/re-oxygenation-induced cell death. H9c2 cells were subjected to OGD (oxygen and glucose deprivation) for 1, 2 or 4 h, followed by re-oxygenation with medium containing normal oxygen (21%) for 24 h. MTT results demonstrated that OGD/re-oxygenation significantly inhibited H9c2 cell viability, which was alleviated by salidroside (10 μM) (Fig. 1A). The cytoprotective effect of salidroside was dose-dependent, salidroside at doses between 10 and 50 μM dramatically inhibited OGD/re-oxygenation-induced H9c2 cell viability loss (Fig. 1B). As shown in Fig. 1C and D, salidroside (10–50 μM) reduced OGD/re-oxygenation-induced H9c2 cell death, the latter was detected by PI FACS assay. Interestingly, we failed to observe a significant H9c2 cell apoptosis after OGD/re-oxygenation, the latter was detected by Annexin V FACS assay (Fig. 1E) and Caspase-3 activity assay (Fig. 1F). While doxorubicin ("dox") induced obvious H9c2 cell apoptosis (Fig. 1E and F). Necrostatin-1, a necrosis inhibitor, but not apoptosis inhibitors z-VAD-fmk and z-DVED-fmk suppressed OGD/re-oxygenation-induced cytotoxicity (Fig. 1G). Together, these results show that salidroside inhibits OGD/re-oxygenation-induced H9c2 cell necrosis.

3.2. Salidroside inhibits OGD/re-oxygenation-induced ROS production, mitochondrial Cyp-D/p53 association and MMP decrease in H9c2 cells

Studies have demonstrated that OGD/re-oxygenation and ROS induce p53 mitochondrial translocation to form a complex with cyclophilin D (Cyp-D), which regulates mPTP opening to promote cell necrosis [15,20]. Inhibition of this complexation by Cyp-D inhibitor cyclosporine A (CsA), or by Cyp-D/p53 deficiency dramatically inhibited cell necrosis by a number of stimuli [15,21–25]. In this study, through the mito-IP assay, we observed that OGD/re-oxygenation induced p53 mitochondrial translocation (mito-inputs of Fig. 2A) and Cyp-D complexation (mito-IP of Fig. 2A) in

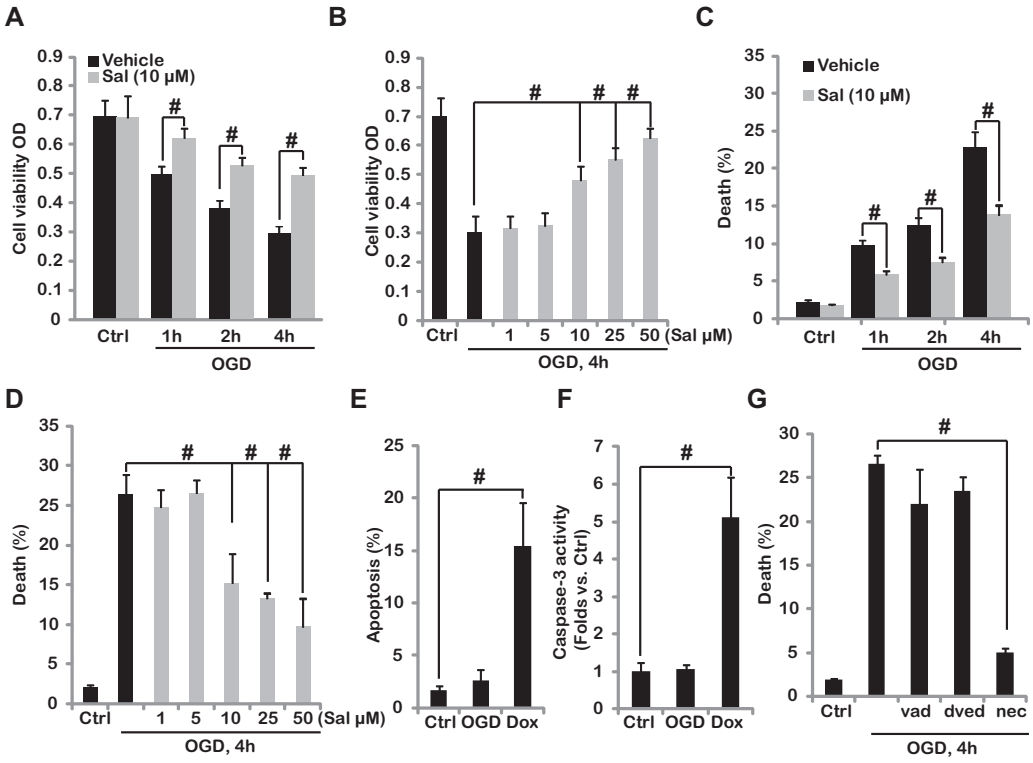

Fig. 1. Salidroside inhibits OGD/re-oxygenation-induced H9c2 cell necrosis-H9c2 cells were pretreated with indicated concentration of salidroside (0–50 μM, 1 h pretreatment), and were maintained under OGD for indicated time, followed by 24 h of re-oxygenation, cell viability and cell death were detected by MTT assay (A and B) and PI FACS assay (C and D), respectively. Apoptosis of H9c2 cells with 3 h OGD or doxorubicin ("dox", 0.1 μM) was analyzed by Annexin V FACS assay (E) or caspase-3 activity assay (F) after 24 h. H9c2 cells were pretreated with z-VAD-fmk ("vad", 40 μM), z-DVED-fmk ("dved", 40 μM), or necrostatin-1 ("nec", 10 μM) for 1 h, and were maintained under OGD for 4 h, followed by 24 h of re-oxygenation, cell viability was tested by MTT assay (G). Data were expressed as mean + SD, experiments were repeated three times, and similar results were obtained. "Ctrl" stands for untreated control group. $^{\#}p < 0.01$.

H9c2 cells, which were largely inhibited by salidroside and anti-oxidant control NAC. Further, both salidroside and NAC alleviated OGD/re-oxygenation-induced ROS production in H9c2 cells (Fig. 2B). Significantly, OGD/re-oxygenation-induced MMP loss, an indicator of mPTP opening, was also alleviated by salidroside and NAC (Fig. 2C). As expected, NAC inhibited OGD/re-oxygenation-induced H9c2 cell death (Data not shown). Thus, these results indicate that OGD/re-oxygenation induces ROS production to cause

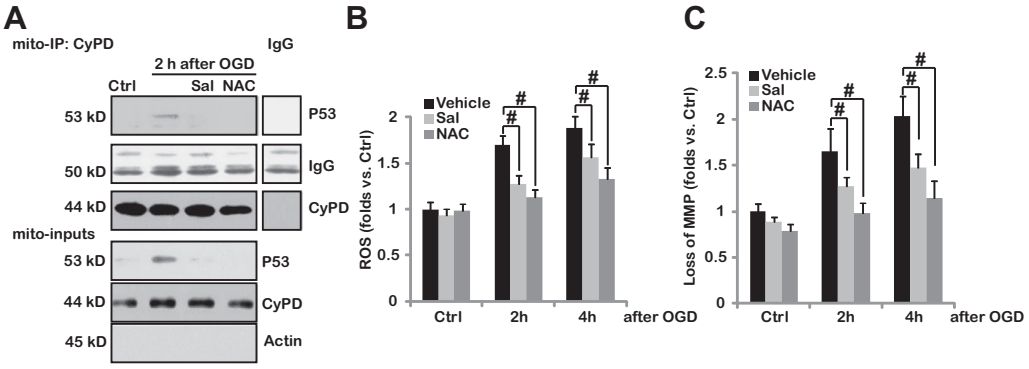

Fig. 2. Salidroside inhibits OGD/re-oxygenation-induced ROS production, mitochondrial Cyp-D/p53 association and MMP decrease in H9c2 cells-H9c2 cells were pre-treated with NAC (400 μM, 1 h pretreatment) or salidroside (10 μM, 1 h pretreatment), and were maintained under OGD for 4 h, followed by re-oxygenation for indicated time, Cyp-D/p53 expression (inputs) and complexation (IP) in mitochondrial was analyzed (A), ROS production was analyzed by FACS assay (B), and MMP was tested by JC-10 assay (C). Data were expressed as mean + SD, experiments were repeated three times, and similar results were obtained. "Ctrl" stands for untreated control group. $^{\#}p < 0.01$.

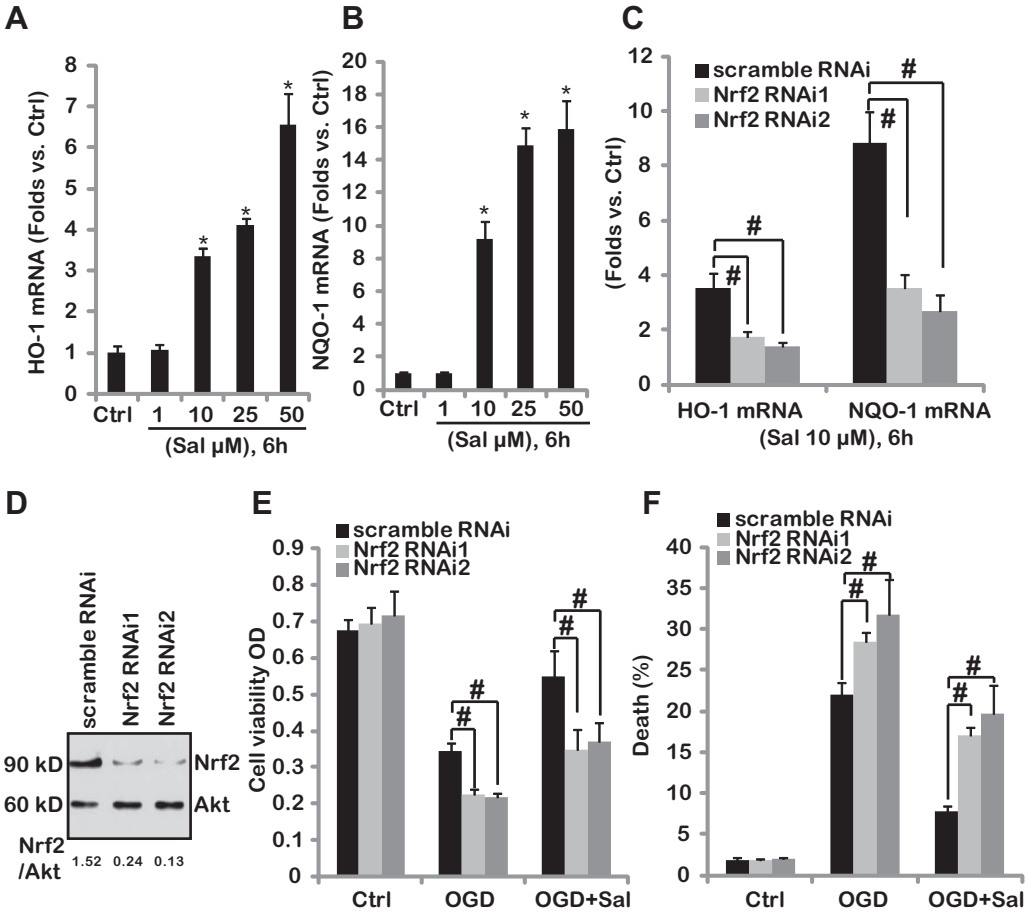

Fig. 3. Salidroside activates Nrf2 signaling in H9c2 cells-H9c2 cells were treated with indicated concentration of salidroside (0–50 μM) for 6 h, mRNA expression of HO-1 and NQO-1 was tested by real-time PCR (A and B). Expression of Nrf2 and Akt (loading) in H9c2 cells infected with scramble shRNA or Nrf2 shRNA-1/-2 lentivirus was tested by Western blots (D). Above cells were treated with salidroside (10 μM) for 6 h, mRNA expression of HO-1 and NQO-1 was tested by real time-PCR (C). Scramble shRNA or Nrf2 shRNA-1/-2 infected H9c2 cells were pre-treated with salidroside (10 μM) for 1 h, and were maintained under OGD for 4 h, followed by re-oxygenation for 24 h, cell viability (E) and cell death (F) were tested by MTT assay and PI FACS assay respectively. Data were expressed as mean + SD, experiments were repeated three times, and similar results were obtained. "Ctrl" stands for untreated control group. Nrf2 (vs. Akt) was quantified (C). $^{*}p < 0.01$ vs. "Ctrl" group (A and B). $^{\#}p < 0.01$.

mitochondrial Cyp-D/p53 complexation, which opens mPTP and promotes H9c2 cell necrosis. Salidroside inhibits OGD/re-oxygenation-induced ROS production, Cyp-D/p53 complexation and mPTP opening, and following H9c2 cell necrosis (Figs. 1 and 2).

3.3. Salidroside activates Nrf2 signaling in H9c2 cells

Antioxidant-responsive element (ARE) regulates transcription of many anti-oxidant genes (i.e. heme oxygenase-1 (HO-1) and quinone oxidoreductase 1 (NQO-1)) and phase II detoxification enzymes [26,27]. Nrf2 regulates transcriptional activation of above genes through binding to ARE [28]. Thus, Nrf2-ARE-activated genes neutralize oxidative stresses and protect cells from many stresses [26]. Above results have shown that salidroside inhibited OGD/re-oxygenation-induced ROS production. We thus tested salidroside effect on Nrf2 signaling. Real-time PCR results showed that salidroside dose-dependently increased mRNA expression of Nrf2-regulated genes HO-1 (Fig. 3A) and NQO-1 (Fig. 3B). Knockdown of Nrf2 through two non-overlapping shRNAs (Fig. 3D) significantly inhibited OGD/re-oxygenation-induced NQO-1/HO-1 mRNA expression (Fig. 3C), indicating that salidroside activates Nrf2 signaling to promote transcription of ARE-genes (NQO-1/HO-1). Importantly, salidroside-mediated pro-survival effect against OGD/re-oxygenation was also inhibited by Nrf2 knockdown (Fig. 3E and F). Thus, these results suggest that salidroside activates Nrf2 to inhibit OGD/re-oxygenation-induced H9c2 cell death.

3.4. Akt inhibitors suppress salidroside-induced transcription of Nrf2-regulated genes and pro-survival effect in H9c2 cells

Recent studies have demonstrated that Akt signaling plays an important role in Nrf2 activation [29–32]. Next, we tested whether Akt was important for salidroside-induced Nrf2 activation. Western blot results in Fig. 4A demonstrated that salidroside (10 μM) induced significant Akt activation (phosphorylate at Ser 473 and Thr 308) in H9c2 cells, which was blocked by Akt inhibitors LY 294002 and wortmannin. Both inhibitors suppressed salidroside-induced mRNA expression of Nrf2-regulated genes (NQO-1/HO-1) (Fig. 4B and C). Importantly, salidroside-mediated pro-survival effect against OGD/re-oxygenation in H9c2 cells was also alleviated by LY 294002 and wortmannin (Fig. 4D and E). These results indicate that salidroside-induced Nrf2 signaling activation and

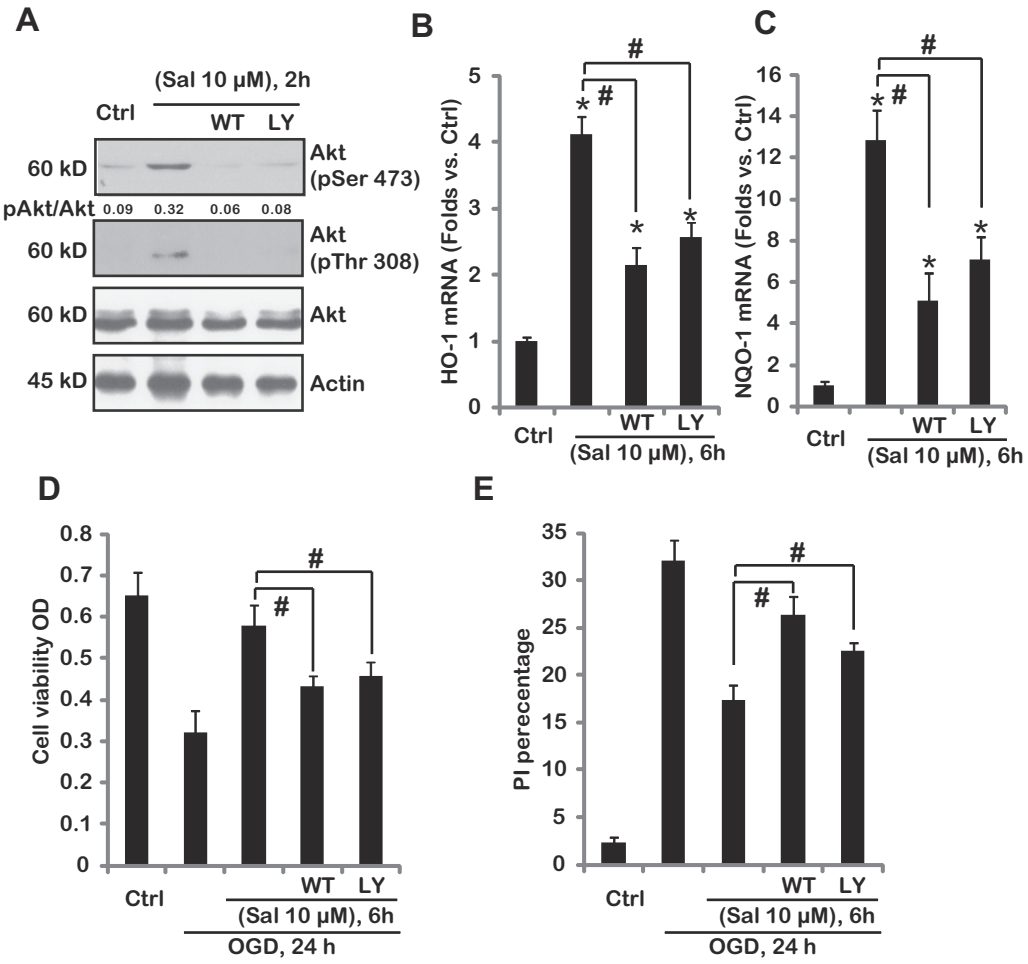

Fig. 4. Akt inhibitors suppress salidroside-induced transcription of Nrf2-regulated genes and pro-survival effect in H9c2 cells-H9c2 cells were pre-treated with LY 294002 (LY, 1 μM) and wortmannin (WT, 1 μM) for 1 h, followed by salidroside (10 μM) stimulation for 2 h, expression of indicated proteins was tested by Western blots (A), mRNA expression of HO-1 and NQO-1 was tested by real time-PCR (B and C). H9c2 cells were pre-treated with LY 294002 (LY, 1 μM) and wortmannin (WT, 1 μM) for 1 h, followed by salidroside (10 μM, 1 h) stimulation, cells were then maintained under OGD for 4 h, followed by 24 h of re-oxygenation, cell viability and cell death were detected by MTT assay (D) and PI FACS assay (E), respectively. Data were expressed as mean + SD. Experiments were repeated three times, and similar results were obtained. P-Akt Ser 473 (vs. Akt) was quantified (A). $^{*}p < 0.01$ vs. "Ctrl" group (B and C). $^{\#}p < 0.01$.

cytoprotective effect again OGD/re-oxygenation are dependent on Akt activation in H9c2 cells.

4. Discussion

The exact role of p53 in the regulation of cell necrosis is recently established [15]. When facing certain necrotic stresses, cytoplasmic p53 translates to the inner mitochondrial membrane, where it forms a complex with local protein Cyp-D. The complexation will trigger mPTP opening to promote cell necrosis [15,21,33,34]. On the other hand, inhibition of this complexation by Cyp-D inhibitors or by p53/Cyp-D deficiency significantly cell necrosis by various stresses [15,21,23,33,34]. In the current study, We didn't observe significant cell apoptosis by OGD/re-oxygenation in H9c2 cells. Rather, OGD/re-oxygenation induced p53/Cyp-D mitochondrial complexation, MMP loss (mPTP opening) and mainly cell necrosis in H9c2 cells, which were significantly inhibited by salidroside. Thus, we propose a novel cytoprotective mechanism of salidroside: inhibition of programmed necrosis.

Although the anti-oxidant ability of salidroside has been studied, the underlying mechanism is not fully understood. Nrf2 regulates transcriptional activation of anti-oxidant genes through binding with ARE [28]. We found that salidroside increased mRNA expression of Nrf2-regulated genes including HO-1 and NQO-1 in H9c2 cells, which was prevented by Nrf2 knockdown. Significantly, salidroside-mediated pro-survival effect against OGD/re-oxygenation was also inhibited by Nrf2 knockdown. Thus, Nrf2 activation is important for salidroside-mediated anti-oxidant and pro-survival effect.

We found that salidroside activated Akt in H9c2 cells, as reported by other studies [8,35]. It has been shown that Akt signaling is an important regulator for Nrf2 activity. Lee et al. showed that sulforaphane-induced Nrf2 phosphorylation and activation is inhibited by LY 294002 [31]. Similarly, Akt in-activation markedly reduced pyocyanin-stimulated Nrf2 nuclear accumulation and transcriptional activation of ARE-genes [30]. Further, salvianolic acid A-activated Nrf2 signaling is mediated through Akt signaling [29]. We found that LY 294002 and wortmannin, two Akt inhibitors, not only suppressed salidroside-induced HO-1/NQO-1 gene transcription, but also eliminated its cytoprotective effect against OGD/re-oxygenation in H9c2 cells. Based on these data, we suggest that Akt activation is required for salidroside-induced Nrf2 activation and pro-survival effect again OGD/re-oxygenation in H9c2 cells.

In summary, the results of this study suggest that salidroside inhibits OGD/re-oxygenation-induced H9c2 cell necrosis through activating Akt-dependent Nrf2 signaling.

Conflict of interest

The authors have no conflict of interest.

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