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Silibinin activates AMP-activated protein kinase to protect neuronal cells from oxygen and glucose deprivation-re-oxygenation

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ABSTRACT

In this study, we explored the cytoprotective potential of silibinin against oxygen–glucose deprivation (OGD)-induced neuronal cell damages, and studied underling mechanisms. *In vitro* model of ischemic stroke was created by keeping neuronal cells (SH-SY5Y cells and primary mouse cortical neurons) in an OGD condition followed by re-oxygenation. Pre-treatment of silibinin significantly inhibited OGD/re-oxygenation-induced necrosis and apoptosis of neuronal cells. OGD/re-oxygenation-induced reactive oxygen species (ROS) production and mitochondrial membrane potential (MMP) reduction were also inhibited by silibinin. At the molecular level, silibinin treatment in SH-SY5Y cells and primary cortical neurons led to significant AMP-activated protein kinase (AMPK) signaling activation, detected by phosphorylations of AMPKα1, its upstream kinase liver kinase B1 (LKB1) and the downstream target acetyl-CoA Carboxylase (ACC). Pharmacological inhibition or genetic depletion of AMPK alleviated the neuroprotective ability of silibinin against OGD/re-oxygenation. Further, ROS scavenging ability by silibinin was abolished with AMPK inhibition or silencing. While A-769662, the AMPK activator, mimicked silibinin actions and suppressed ROS production and neuronal cell death following OGD/re-oxygenation. Together, these results show that silibinin-mediated neuroprotection requires activation of AMPK signaling.

1. Introduction

Silibinin is the major pharmacologically active compound of the polyphenolic flavonoid extract from the medicinal plant *Silybum marianum*. Silibinin has traditionally been used for the treatment of liver diseases. Hepatoprotection of silibinin has been attributed to its anti-oxidative properties, to inhibition of phosphatidylcholine synthesis, and to stimulation of hepatic synthesis of RNA and proteins [1–3]. However, the underlying mechanisms associated with the cyto-protective ability of silibinin are not fully understood. The neuroprotective activity of this orally active flavonoid has not been extensively studied.

Regular oxygen and glucose supply is critical to maintain normal neuronal functions. Loss of the regular supply, even for a short period, will lead to ischemia/re-oxygenation, reactive oxygen species (ROS) production, eventually causing neuronal cell death and brain damages [4]. Groups have been using *in vitro* ischemic model of oxygen–glucose deprivation (OGD) followed by re-oxygenation to investigate the underlying mechanisms of ischemic neuronal damages, and to explore the possible interventions [5]. Here, we investigated the cytoprotective potential of silibinin against OGD/re-oxygenation-induced neuronal cell damages, and studied underling mechanisms by focusing on AMP-activated protein kinase (AMPK) signaling.

AMPK is a master regulator of ATP homeostasis and energy metabolism [6]. It is also important for regulating cell apoptosis or survival under stress conditions. However, whether AMPK is pro-apoptotic or pro-survival is dependent on the type and/or severity of stresses. Severe stresses including vincristine [7,8], taxol [9,10] and temozolomide [11]-activated AMPK appears pro-apoptotic. While in conditions like starvation [12], hypoxia [13] and oxidative stress [14], AMPK activation could promote cell survival. The pro-survival property following AMPK activation is mediated through regulating its downstream targets. Activated AMPK maintains nicotinamide adenine dinucleotide phosphate (NADPH) homeostasis and rescues cells from oxidative stresses [12]. With energy starvation, AMPK activation phosphorylates tuberous

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sclerosis complex 2 (TSC2) to in-activate mammalian target of rapamycin (mTOR), thus promoting cell survival [15]. AMPK could also activate cyto-protective autophagy [14,16,17] though direct (Ulk1 phosphorylation) [18] or indirect (mTOR inhibition) mechanisms [18]. In this study, we found that silibinin activates AMPK signaling to rescue neuronal cells from OGD/re-oxygenation.

2. Materials and methods

2.1. Reagents and chemicals

Silibinin (Sigma, St. Louis, MO) was dissolved in DMSO. The final concentration of DMSO in the culture medium did not exceed 0.1% (v/v), same amount of DMSO was added in control cultures. Compound C and A-769662 were purchased from Sigma. Anti-AMPKα1, tubulin and acetyl-CoA carboxylase (ACC) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-p-AMPKα1 (Thr 172), p-ACC (Ser 79), p-liver kinase B1 (LKB1) (Ser-428) and LKB1 antibodies were purchased from Cell Signaling Tech (Denver, MA).

2.2. SH-SY5Y cell culture

Neuroblastoma SH-SY5Y cells were grown in DMEM with 10% fetal bovine serum (FBS, Hyclone, Suzhou, China) and 1% penicillin–streptomycin (Gibco) at standard culture conditions.

2.3. Primary culture of mouse cortical neurons

Neocortical cultures of neurons from mouse embryos at 14–15 d of embryonic development were prepared, cerebral cortices were micro-dissected in DMEM, carefully stripped off the meninges and mechanically dissociated to obtain single cell suspension. The cells were plated at a density of 0.75×10^6 per well in 24-well dishes, pre-coated with poly-D-lysine (0.1 mg/mL, Sigma) and laminine (0.02 mg/mL, Invitrogen, Shanghai, China), in DMEM supplemented with 10% FBS and 2 mM glutamine (Sigma). Following 2 h of culture, culture medium was removed and switched to DMEM supplemented with 2 mM glutamine and 1% N_2 defined Supplement (Invitrogen). Cultures were then maintained for 8–10 d in a humidified incubator (5% CO_2, 37 °C).

2.4. Oxygen–glucose deprivation (OGD)-re-oxygenation

SH-SY5Y cells or primary cortical neurons 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 different periods to produce oxygen deprivation and then re-oxygenated (returned to the normal aerobic environment). Experimental parameters were assayed at 6–24 h following re-oxygenation.

2.5. Cell viability assay

Cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, neuronal cells were seeded in 96-well plates at a density of $1–2 \times 10^4$ cells/well. After treatment, MTT tetrazolium salt (Sigma, 0.20 mg/mL) was added to each well, cells were further incubated in CO_2 incubator for 2–3 h. DMSO (200 μL/well) was then added to dissolve formazan crystals, the absorbance of each well was observed by a plate reader at a wavelength of 450 nm.

2.6. Apoptosis assay by flow cytometry

After treatment, cells were detached and incubated in 500 μL binding buffer and 5 μL annexin V-FITC (Invitrogen) at room temperature for 15 min in the dark. Cell apoptosis was detected by sorting Annexin V-FITC cells through fluorescence-activated cell sorting (FACS) with a C6 Becton–Dickinson Machine. Percentage of apoptosis was calculated by Annexin V ratio.

2.7. Caspase-3 activity assay

Caspase-3 activity was determined using the ApoAlert CPP32 Assay kit according to the manufacturer's instructions (Clontech, Palo Alto, CA). These assays depended on the cleavage of a specific colorimetric caspase substrate, DEVD-ρNA (Asp-glu-val-asp-ρ-nitroanilide). After treatment, cells were collected by scraping in cold PBS, centrifuged and lysed in the cell lysis buffer provided on ice for 10 min. Extracts (20 μg/sample) were reacted with an equal volume of 2× reaction buffer containing DTT (10 mM) and the colorimetric caspase-3 substrate (DEVD-ρNA). Mixtures were maintained in a water bath at 37 °C for 45 min and then analyzed in a spectrophotometer at 405 nm.

2.8. Mitochondrial membrane potential detection

Estimation of mitochondrial membrane potential (MMP) was performed using JC-10 (final concentration 5 μM) (Enzo Life Sciences, Farmingdale, USA), a membrane permeable fluorescent probe. JC-10 accumulates and aggregates in mitochondria, selectively generating an orange emission profile (λ 590 nm). As membrane potential decreases, JC-10 monomers are generated, resulting in a shift to green fluorescence emission (λ 485 nm). Decrease of MMP was then expressed as the intensity of green fluorescence, which was detected by a Fluoroskan system (Thermo-Labsystems, Helsinki, Finland) with an excitation filter of 485 nm.

2.9. ROS assay

After treatment, ROS level in neuronal cells was determined with 2′,7′-dichlorofluorescin diacetate (H2-DCFDA, Molecular Probes, Shanghai, China), a reactive oxygen species-sensitive dye, based on protocols provided. The nonpolar, nonionic H2-DCFDA crossed cell membranes and is hydrolyzed into non-fluorescent H2-DCF by intracellular esterase. In the presence of ROS, H2-DCF is rapidly oxidized to become highly fluorescent DCF.

2.10. Western blots

Cell lysates were prepared with lysis buffer (Sigma). 30–40 μg of protein lysates per sample were denatured in 5× SDS–PAGE sample buffer and subjected to SDS–PAGE gels. The separated proteins were transferred onto PVDF membranes followed by blocking with 10% non-fat milk (w/v) in PBST (PBS and 0.1% Tween 20) for 1 h at room temperature. Membranes were then probed with specific primary antibodies followed by peroxidase-conjugated secondary antibody and visualized by ECL detection system.

2.11. Generating LKB1 knocked-down SH-SY5Y cells

Two sets of lentiviral particles encoding LKB1 shRNAs were purchased form Sigma (SHCLNG-NM_000455, LKB1-shRNA-a) or Santa Cruz (sc-35816-SH, LKB1-shRNA-b). SH-SY5Y cells were infected with lentiviral particles encoding non-target scramble shRNA (Santa

Cruz) or LKB1-shRNA (-a/-b), 24 h after infection, virus-infected cells were selected by puromycin (1.0 μg/mL) for 4 d. Stable Clones were screened for reduced expression of LKB1 (Western blots).

2.12. AMPKα1 shRNA

Sequences targeting of human AMPKα1 were sub-cloned into pSUPER-puro plasmid (OligoEngine, Seattle, WA). Plasmids were transfected with pVSVG (Addgene, Cambridge, MA) into HEK-293 cells for lentivirus packaging. Sub-confluent SH-SY5Y cells were then infected with the resultant lentivirus. Selection and maintenance of stable SH-SY5Y cell clones were performed in DMEM containing 1 μg/mL puromycin. Stable clones were screened for reduced expression of AMPKα1. The sequences chosen for targeting AMPKα1 subunit were from literatures: *AMPKα1-shRNA-a* [19] and *AMPK α1-shRNA-b* [20].

2.13. Transit knockdown of AMPKα1 in primary cortical neurons

Primary cells were transfected with 100 nM of mouse AMPKα1 small interfering RNA (siRNA, sc-29674, Santa Cruz) using Lipofectamine 2000 (Invitrogen). After 6 h of siRNA incubation, cells were cultured in fresh medium and incubated for additional 48 h. The efficacy of siRNA was confirmed by reduced expression of AMPKα1 by Western blot analysis.

2.14. Statistical analysis

All experiments were repeated at least three times, and similar results were obtained. The data were analyzed using the Jandel Scientific SigmaStat 2.03 software. For all measurements Student's *t* test was employed to assess the statistical significance of difference between two groups if needed. Where the *p*-value was <0.05, groups were considered to be significantly different.

3. Results

3.1. Silibinin inhibits oxygen glucose deprivation (OGD)/re-oxygenation-induced neuronal cell death

We first examined the *in vitro* activity of silibinin in cultured SH-SY5Y cells. MTT cell viability results demonstrated that treatment of silibinin inhibited SH-SY5Y cell survival only at relatively high concentrations (100–200 μM) (Fig. 1A). These results are not surprisingly, as silibinin is being investigated as an effective anticancer drug [21]. Significantly, non-cytotoxic silibinin (10–50 μM) dose-dependently inhibited OGD/re-oxygenation-induced SH-SY5Y cell death, detected by viability reduction (Fig. 1B) and LDH release (Fig. 1C). Silibinin at 50 μM demonstrated the most significant cyto-protective effect, which inhibited SH-SY5Y viability decrease (Fig. 1D) and death (LDH release, Fig. 1E) induced by varying degrees of OGD/re-oxygenation. In primary cultured cortical neurons, OGD/re-oxygenation also induced cell death, pretreatment with silibinin (50 μM) again inhibited OGD/re-oxygenation-induced neuronal viability decrease (Fig. 1F) and LDH release (Fig. 1G). Together, these results show that silibinin protect neuronal cells from OGD/re-oxygenation insults.

3.2. Silibinin inhibits OGD/re-oxygenation-induced ROS production, mitochondrial membrane potential decrease and apoptosis in neuronal cells

Above results have showed the neuroprotective ability of silibinin. Then we tested its role on cell apoptosis. OGD/re-oxygenation

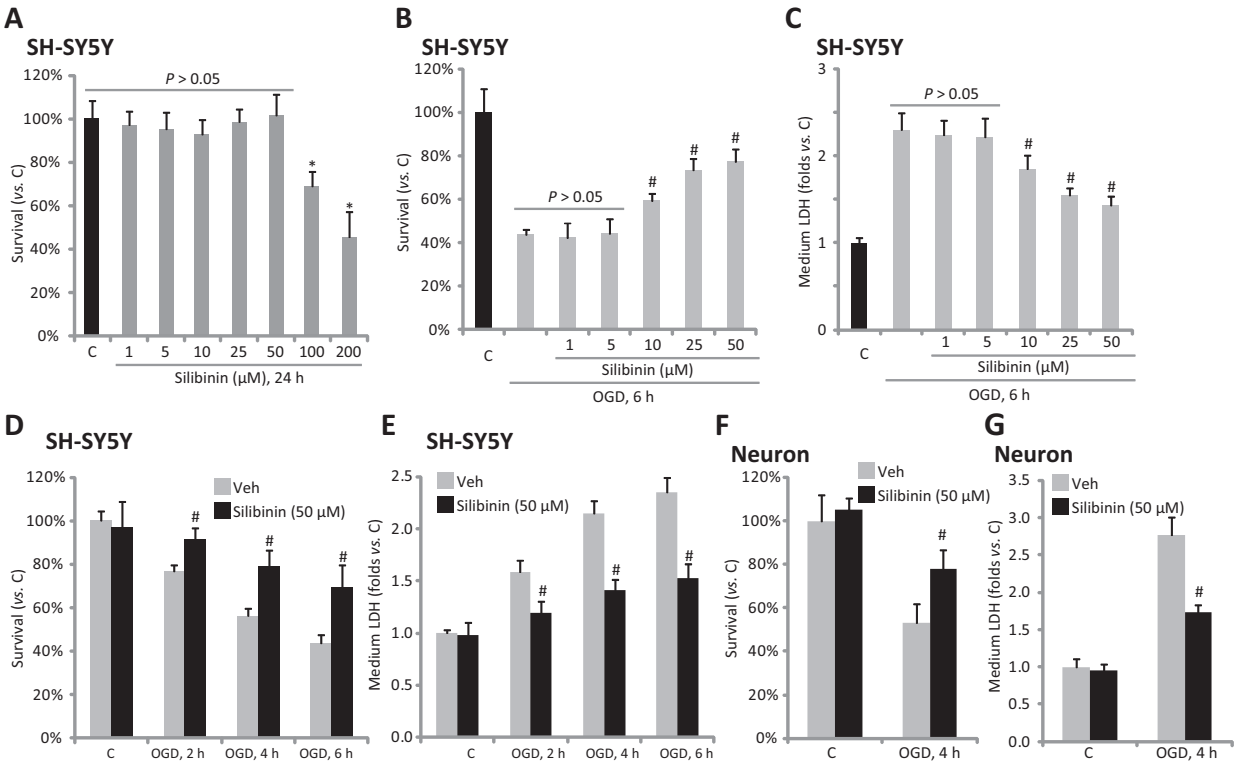

Fig. 1. Silibinin inhibits oxygen glucose deprivation (OGD)/re-oxygenation-induced neuronal cell death. The viability of SH-SY5Y cells with indicated silibinin (1–200 μM, 24 h) treatment was tested by MTT assay (A). SH-SY5Y cells (B–E) or primary cortical neurons (F and G) were pretreated with indicated concentration of silibinin (1–50 μM, 1 h pretreatment), and were maintained under OGD for indicated time (2–6 h), followed by 24 h of re-oxygenation, cell viability was tested by MTT assay (B, D and F), cell death was tested by LDH release (C, E and G). "C" stands for DMSO control group (Same for all figures). Experiments in this and following figures were repeated three-four times with similar results obtained. Data were presented as mean ± SD (for all figures). $^{*}p < 0.05$ vs. "C" group (A). $^{\#}p < 0.05$ vs. OGD/re-oxygenation only group (B–G).

activated apoptosis in SH-SY5Y cells, evidenced by increased caspase-3 activity and Annexin V percentage (Fig. 2A and B). Importantly, pretreatment with silibinin dose-dependently inhibited SH-SY5Y cell apoptosis by OGD/re-oxygenation. Silibinin at concentration of 50 μM showed highest efficiency (Fig. 2A and B). Since ROS production and subsequent mitochondrial permeability transition pore (mPTP) opening are main mediators of OGD/re-oxygenation-induced damages [5], we tested the status of these parameters in silibinin-treated cells. In SH-SY5Y cells, OGD/re-oxygenation led to ROS production (Fig. 2C) and mitochondrial membrane potential (MMP) decrease (indicator of mPTP opening) (Fig. 2D), which were dramatically inhibited by silibinin pretreatment (Fig. 2C and D). In primary cortical neurons, apoptosis activation (Fig. 2E) and ROS production (Fig. 2F) by OGD/re-oxygenation were also largely inhibited by silibinin pretreatment.

3.3. Silibinin activates LKB1–AMPK–ACC signaling in neuronal cells

We aimed to test the involvement of AMPK signaling in silibinin-mediated neuroprotection. We first examined the status of AMPK signaling in silibinin-treated cells. Western blot results in Fig. 3A demonstrated that silibinin induced significant AMPK activation in SH-SY5Y cells, detected by phosphorylations of AMPKα1 (Thr-172) and its downstream target acetyl-CoA Carboxylase (ACC, Ser 79). There are several potential upstream kinases for AMPK have been indentified including the best-characterized liver kinase B1 (LKB1), as well as ataxia telangiectasia mutated (ATM), Ca^{2+}/Calmodulin-dependent Kinase Kinase-β (CaMKK-β) and transforming growth factor-β (TGF-β) activated kinase 1 (TAK1) [22]. Here we found that LKB1 was activated (phosphorylation at Ser 428) by silibinin (Fig. 3A). Importantly, partial depletion of LKB1 through shRNAs dramatically inhibited silibinin-induced AMPK activation (Fig. 3B), indicating that LKB1 served as the upstream kinase for AMPK activation by silibinin. Note that we utilized two shRNAs targeting non-overlapping cDNA sequence of LKB1, with similar results obtained (Fig. 3B). Significantly, as shown in Fig. 3C, OGD/re-oxygenation also induced moderate LKB1–AMPK signaling activation in SH-SY5Y cells, which was dramatically enhanced by silibinin. Thus, silibinin enhanced AMPK activation by OGD/re-oxygenation. In primary cortical neurons, LKB1–AMPK was activated by silibinin (Fig. 3D). Together, these results show that silibinin activates LKB1–AMPK–ACC signaling in neuronal cells.

3.4. Activation of AMPK is important for silibinin-mediated neuroprotection

To explore the role of AMPK in silibinin-mediated neuroprotective effects, pharmacological and genetic means were applied. OGD/re-oxygenation-induced SH-SY5Y damages, evidenced by viability decrease (Fig. 4A) and apoptosis induction (Fig. 4B), were again inhibited by silibinin. The AMPK inhibitor compound C ("CC") inhibited cyto-protection by silibinin (Fig. 4A and B). While A-769662 ("A-7"), the AMPK activator, mimicked silibinin actions and inhibited OGD/re-oxygenation-induced SH-SY5Y damages (Fig. 4A and B). Further, the anti-oxidant capability of silibinin against OGD was also alleviated by compound C (Fig. 4C). On the other hand, A-769662 decreased ROS accumulation after OGD/re-

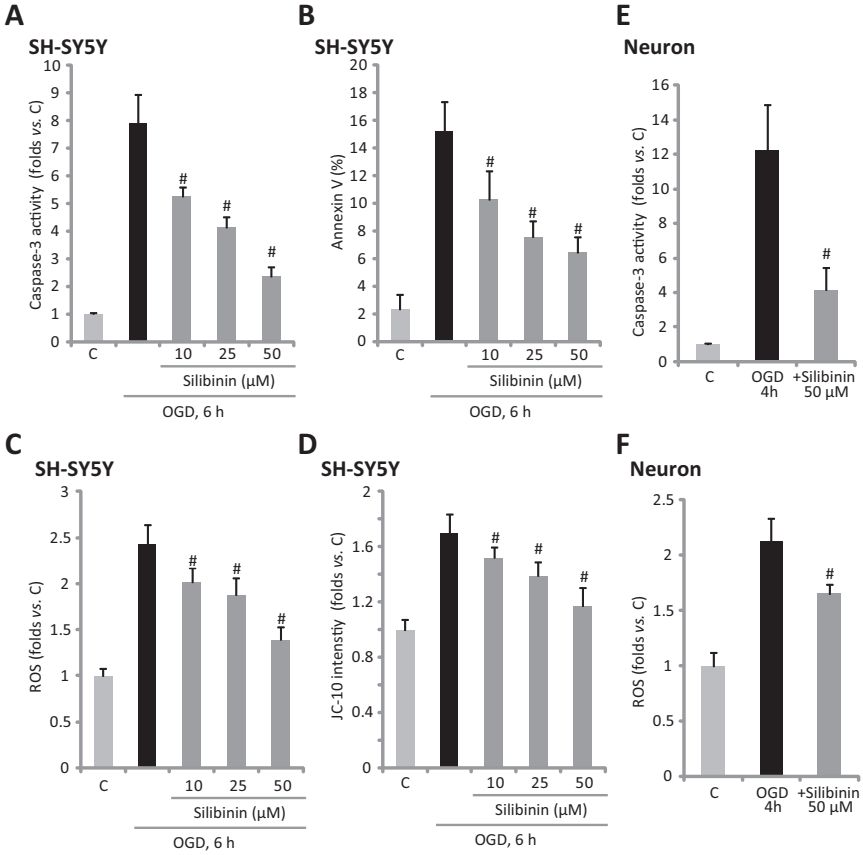

Fig. 2. Silibinin inhibits OGD/re-oxygenation-induced ROS production, mitochondrial membrane potential decrease and apoptosis in neuronal cells. SH-SY5Y cells (A–D) or primary cortical neurons (E and F) were pretreated with indicated concentration of silibinin (10–50 μM, 1 h pretreatment) and were maintained under OGD for indicated time (4/6 h), 24 h after re-oxygenation cell apoptosis was detected by caspase-3 activity assay (A and E) and Annexin V FACS assay (B), ROS production (C and F) and MPP reduction (D) were also tested 6 h after re-oxygenation. $^{\#}p < 0.05$ vs. OGD only group.

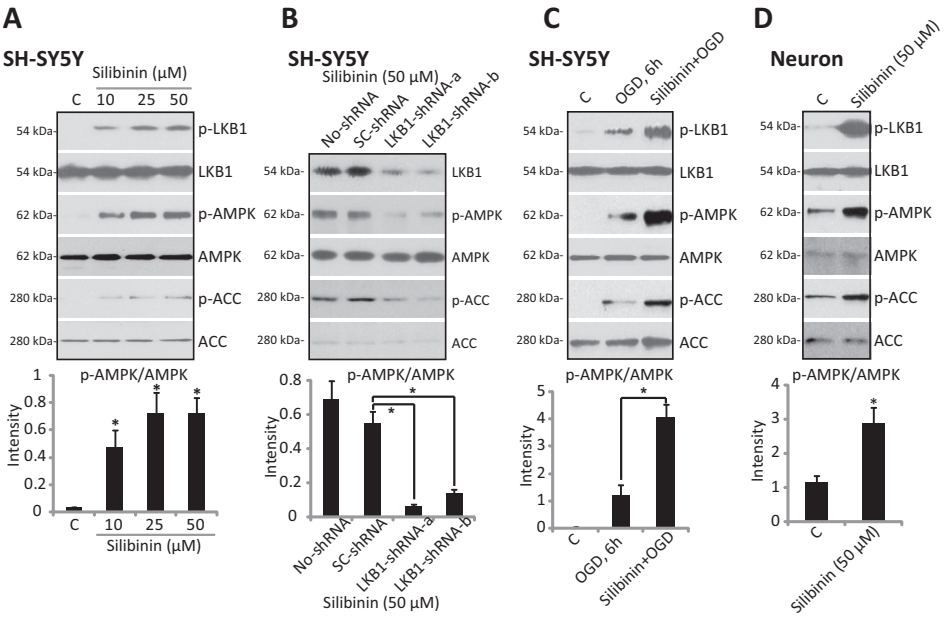

Fig. 3. Silibinin activates LKB1–AMPK–ACC signaling in neuronal cells. SH-SY5Y cells (A) or primary cortical neurons (D) were treated with indicated concentration of silibinin (10–50 μM) for 3 h, phosphorylations of AMPKα1 (Thr-172), ACC (Ser-79) and LKB1 (Ser-428), as well as their non-phosphorylated kinases were tested by Western blots. Parental SH-SY5Y cells or SH-SY5Y cells infected with viral particles (10 μL/mL) containing scramble control shRNA (SC-shRNA), LKB1-shRNA-a or LKB1-shRNA-b were treated with silibinin (50 μM) for 3 h, LKB1–AMPK–ACC signaling was tested (B). SH-SY5Y cells were pretreated with silibinin (50 μM, 1 h pretreatment), and were maintained under OGD for 6 h, after 3 h of re-oxygenation, LKB1–AMPK–ACC signaling activation was tested (C). p-AMPK was quantified. $^{*}p < 0.05$ vs. "C" group (A and D). $^{*}p < 0.05$ (B and C).

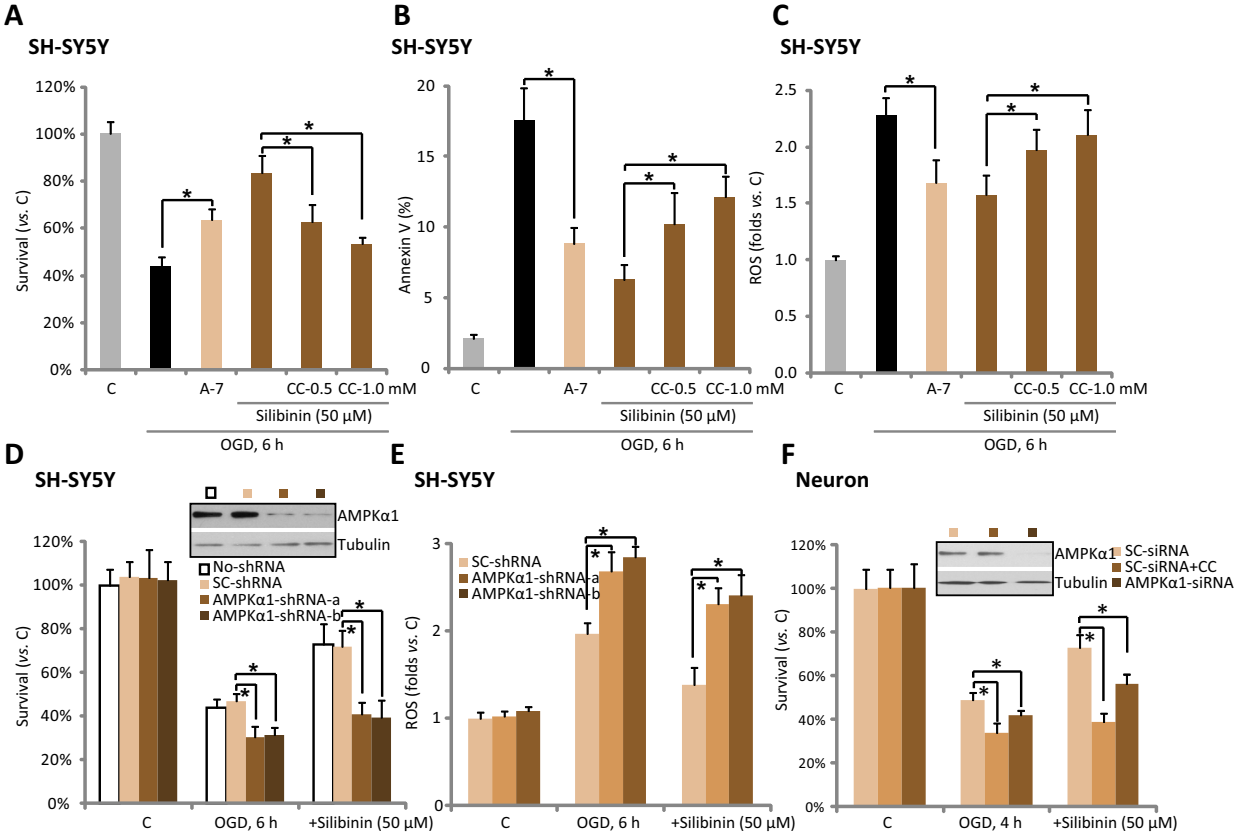

Fig. 4. Activation of AMPK is important for silibinin-mediated neuroprotection. SH-SY5Y cells were pre-treated with AMPK activator A-769662 ("A-7", 10 μM) or silibinin (50 μM) for 1 h, in the presence or absence of Compound C (CC, 0.5/1.0 mM), followed by OGD for 6 h, 24 h after re-oxygenation cell viability and cell apoptosis were tested by MTT assay (A) and Annexin V assay (B), respectively; ROS production was tested 6 h after re-oxygenation (C). Stable SH-SY5Y cells expressing indicated shRNA (scramble control shRNA, AMPKα1-shRNA-a, AMPKα1-shRNA-b) or their parental cells were maintained under OGD (6 h), with or without silibinin (50 μM, 1 h) pretreatment, cells were re-oxygenized, cell viability (D, lower panel) and ROS production (E) were tested after re-oxygenation. Expressions of AMPKα1 and tubulin were shown (D, upper panel). Primary cortical neurons transfected with scramble siRNA or AMPKα1 siRNA were pre-treated silibinin (50 μM) for 1 h, in the presence or absence of Compound C (CC, 1.0 mM), followed by OGD for 4 h and 24 h re-oxygenation, cell viability was tested (F, lower panel), expressions of AMPKα1 and tubulin were shown (F, upper panel). $^{*}p < 0.05$.

oxygenation (Fig. 4C). These results indicate that activation of AMPK is important for silibinin-mediated ROS scavenging and neuroprotection.

To further support this hypothesis, shRNA strategy was applied to selectively knockdown AMPKα1 in SH-SY5Y cells. Western blot results showed that AMPKα1 expression was significantly reduced in stable SH-SY5Y cells expressing AMPKα1-shRNAs (-a/-b, Fig. 4D, upper panel). Silibinin-mediated neuroprotection was largely inhibited in AMPKα1-depleted cells (Fig. 4D, lower panel). Again we utilized two non-overlapping shRNAs against AMPK. Significantly, in the absence of silibinin, OGD/re-oxygenation damage was aggravated with AMPKα1 knockdown. In another word, AMPKα1 depletion could increase the severity of OGD/re-oxygenation in SH-SY5Y cells. Thus, OGD/re-oxygenation-induced AMPK activation, although moderate (Fig. 3C), exerted a pro-survival role. And this OGD/re-oxygenation-activated AMPK was further enhanced by silibinin (Fig. 3C). Meanwhile, ROS production by OGD/re-oxygenation was also exacerbated in AMPK-depleted cells (Fig. 4E), and silibinin-induced anti-oxidant capacity was dramatically alleviated with AMPKα1 depletion (Fig. 4E).

In neurons, OGD/re-oxygenation-induced damage, detected by viability reduction, was augmented by AMPK inhibitor compound C or by AMPKα1 siRNA (Fig. 4F), and neuroprotection by silibinin was inhibited with AMPK inhibition (Fig. 4F, lower panel). Blot data in Fig. 4F (upper panel) confirmed the efficiency of AMPKα1 siRNA, resulting in substantial AMPKα1 downregulation. These results again confirmed the involvement of AMPK activation in silibinin-mediated neuroprotection.

4. Discussions

OGD/re-oxygenation-induced neuronal cell damage is accompanied with oxidative stress [23,24]. Serve and/or sustained OGD inhibits mitochondrial complex-I activity to interrupt mitochondrial functions, and when coupled with re-oxygenation, ROS will be produced, causing subsequent cell death [23,24]. In the current study, we found that OGD/re-oxygenation-induced ROS production was significantly inhibited by silibinin, which could explain its dramatic neuroprotective activity. We show that AMPK activation is important for silibinin-mediated neuroprotective effect. Inhibition or genetic depletion of AMPK alleviated the neuroprotective activity by silibinin against OGD/re-oxygenation. While A-769662, the AMPK activator, mimicked silibinin' s actions and suppressed OGD/re-oxygenation-induced ROS production and neuronal cell death.

Jeon et al. discovered that AMPK activation, through regulating NADPH maintenance, is important for ROS clearance under energy stress conditions [12]. Activation of AMPK phosphorylates and inhibits its downstream signal ACC, thus decreasing NADPH consumption by inhibiting fatty-acid synthesis and increasing NADPH generation by means of fatty-acid oxidation [12]. Following that study, a number of other groups have confirmed they discoveries, and identified the anti-oxidative property of AMPK [14,25,26]. In this study, our evidence suggests that AMPK activation by silibinin might be responsible for its ROS scavenging ability. Inhibition of AMPK through pharmacological or genetic means almost abolished silibinin-mediated inhibition on ROS production.

It should be noted that OGD/re-oxygenation alone (no silibinin presence) also slighted activated AMPK signaling in neuronal cells (Fig. 3C), and this AMPK activation was also pro-survival (Fig. 4D–F). AMPK inhibition or silencing exacerbated OGD/re-oxygenation-induced ROS production and neuron damages. Significantly, OGD/re-oxygenation-induced pro-survival AMPK activation was dramatically enhanced by pretreatment of silibinin, which exerted strong neuroprotection. Thus, AMPK signaling might be an important drug target for interventions against OGD/re-oxygenation/ischemia damages.

In conclusion, the present study demonstrates that silibinin exhibits a neuroprotective effect against OGD/re-oxygenation through activating AMPK signaling. We have identified the activation of AMPK as the key element in the regulation of neuroprotection by silibinin.

Conflict of interest

There authors have no conflict of interests.

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