c-Abl Tyrosine Kinase Mediated Neuronal Apoptosis in Subarachnoid Hemorrhage by Modulating the LRP-1-Dependent Akt/GSK3β Survival Pathway

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Abstract

Accumulating evidence suggests that neuronal apoptosis plays a critical role in early brain injury (EBI) after subarachnoid hemorrhage (SAH), and the inhibition of apoptosis can induce neuroprotective effects in SAH animal models. c-Abl has been reported to promote neuronal apoptosis in Alzheimer's disease and cerebral ischemia, but its role in SAH had not been illuminated until now. In the present study, the effect of c-Abl on neuronal apoptosis induced by SAH was investigated. c-Abl protein levels and neuronal apoptosis were markedly increased 24 h after SAH, and the inhibition of endogenous c-Abl reduced neuronal apoptosis and mortality and ameliorated neurological deficits. Furthermore, c-Abl inhibition decreased the expression of cleaved caspase-3 (CC-3) after SAH. These results demonstrate the proapoptotic effect of c-Abl in EBI after SAH. Additionally, c-Abl inhibition further enhanced the SAH-induced phosphorylation of Akt and glycogen synthase kinase (GSK)3β. LY294002 abrogated the beneficial effects of targeting c-Abl and exacerbated neuronal apoptosis after SAH. SAH decreased LRP-1 levels and downregulated LRP-1 by RAP and LRP-1 small interfering RNA (siRNA) induced a dramatic decrease in Akt/GSK3β activation in the presence of c-Abl siRNA. This is the first report showing that the c-Abl tyrosine kinase may play a key role in SAH-induced neuronal apoptosis by regulating the LRP-1-dependent Akt/GSK3β survival pathway. Thus, c-Abl has the potential to be a novel target for EBI therapy after SAH.

Highlights

Inhibition of c-Abl reduced neuronal apoptosis and improved neurological deficits in SAH

c-Abl played pro-apoptotic role by inhibiting Akt/GSK3β survival pathway in EBI after SAH

LRP-1 was essential for c-Abl to regulate Akt/GSK3β pathway in SAH

Introduction

Subarachnoid hemorrhage (SAH) caused by aneurysm rupture is a devastating cerebrovascular disease characterized by high mortality and disability. Nearly 60,000 people worldwide suffer from this disease each year[1].Most survivors also have intractable neurological deficits and cognitive dysfunction[2]. Cerebral vasospasm following SAH is widely accepted as the major cause of poor outcomes[3]; however, cumulative data suggest that suppressing vasospasm does not improve patient outcomes[4]. Early brain injury (EBI), defined as a series of pathophysiological changes that occur within the first 72 h after the onset of SAH[5], has now become a focus of research on alleviating injury from SAH[6]. Neuronal apoptosis has been reported to be one of the main pathological mechanisms of EBI after experimental SAH and is observed in human patients with SAH[7,8].Therefore, resistance against neuron apoptosis in the treatment of EBI after SAH has broad research value and application prospects.

c-Abl is an src-related nonreceptor tyrosine kinase that is widely expressed in the nervous system. Recently, numerous intensive studies have focused on the relationship between c-Abl and central nervous

system (CNS) diseases[9].c-Abl has been proven to exert a proapoptotic effect through various mechanisms involved in Parkinson's and Alzheimer's diseases[10,11].To the best of our knowledge, there has been no report on the role of c-Abl in SAH. The Akt/glycogen synthase kinase (GSK)3β signaling pathway was shown to be involved in the neuroprotective mechanism of EBI after SAH, and blockade of the Akt/GSK3β pathway aggravated neuronal apoptosis[12]. There have been few studies exploring whether c-Abl modulates Akt/GSK3β activity in neurons.

Low-density lipoprotein receptor-related protein 1 (LRP-1), a member of the low-density lipoprotein receptor family, is a scavenger receptor involved in endocytosis and signaling receptor that regulates various cellular processes[13].LRP-1, which regulates the Akt survival pathway and insulin pathway in the brain and promotes the antiapoptotic function of neurons, was previously shown to be highly expressed in neurons. Endogenous LRP-1 knockout led to caspase-3 activation and increased neuronal apoptosis[14].In addition, LRP-1 was shown to have a synergistic effect with immunoglobulin on neuronal survival in ischemic stroke, and the LRP-1 antagonist RAP was reported to significantly inhibit the activation of Akt[15]. A study suggested that the inhibition of c-Abl using imatinib increases basal LRP-1-dependent ERK and Akt activation and maintains pancreatic β cell function and survival[16]. However, the role of LRP-1 in regulating Akt expression in neuronal survival after SAH is unclear. The current work investigated the role of c-Abl in SAH and verified that the underlying mechanism of this role may be related to the LRP-1-dependent Akt/GSK3β survival pathway for the first time.

Materials And Methods

Animals

All animal experiments were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Harbin Medical University and strictly complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. A total of 207 male Wistar rats (260-280 g) were purchased from Changchun Yisi Company, which were fed with adequate food and water in a 12/12 hours light/dark room with humidity control and a temperature of 25℃.

SAH model and study design

An SAH rat model was established by endovascular puncture as previously reported[17].Pentobarbital (40 mg/kg) was injected intraperitoneally to anesthetize the animals. The animals were placed in a supine position, and a median incision was made in the neck to expose the common carotid artery and its branches. A single-stranded nylon thread penetrated the external carotid artery and encountered resistance after entering the internal carotid artery, indicating that it had reached the distal bifurcation of the internal carotid artery. The thread was then advanced approximately 3 mm beyond the resistance point and immediately withdrawn, allowing reperfusion of the internal carotid artery. The same operation was performed on rats in the sham group, except the vessels were not punctured.

24 rats were used to detect c-abl expression at 24, 48 and 72 hours after SAH by western blot(n=6).To test the role of c-Abl in SAH, 48 rats were randomly divided into four groups as follows: the sham (n=12), SAH (n=12), SAH+scramble small interfering RNA (siRNA) (n=12), and SAH+c-Abl siRNA (n=12) groups. c-Abl siRNA and scramble siRNA (500 pmol/5 μL) were injected intracerebroventricularly 24 h before SAH. Neuron apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), and levels of the c-Abl, p-Akt, p-GSK3β and cleaved caspase-3 (CC-3) proteins were measured by Western blotting. The neurological function score and SAH severity were also evaluated. The PI3K/Akt inhibitor LY294002 was used to explore the underlying mechanism of the role of c-Abl in SAH-induced apoptosis. A total of 36 rats were randomly divided into three groups as follows: the SAH+scramble siRNA+vehicle (n=12), SAH+c-Abl siRNA+vehicle (n=12), and SAH+c-Abl siRNA+LY294002 (n=12) groups. LY294002 (5 mmol/L, 0.5 ml/animal) in a physiological saline solution was injected into the femoral vein 1 h before SAH induction, and rats in the vehicle group were given the same amount of physiological saline. Western blotting was used to detect protein levels. TUNEL was used to test apoptosis. To investigate the role of LRP-1 in the mechanism by which c-Abl regulates Akt/GSK3β, 54 rats were randomly divided into the following groups: the sham, SAH+vehicle (or scramble siRNA) (n=6), SAH+RAP (or LRP-1 siRNA) (n=6), SAH+c-Abl siRNA+vehicle (or scramble siRNA) (n=6), and SAH+c-Abl siRNA+RAP (or LRP-1 siRNA) (n=6) groups. RAP (0.7 nmol/g body weight) in a physiological saline solution was injected into the femoral vein 1 h before SAH operation, and rats in the vehicle group were given the same amount of physiological saline. Western blotting was conducted to detect protein levels.

Intracerebroventricular injection

Intracerebroventricular injection was conducted as previously reported[18].The rats were anesthetized and placed in stereotactic devices. A 10-μL Hamilton syringe (Hamilton Company, Reno, NV, USA) was inserted into the left ventricle through a burr hole made at the following coordinates: 1.5 mm posterior and 1.0 mm lateral to the bregma and 3.2 mm below the horizontal plane. siRNA in solution was administered at a rate of 0.5 μL/min 24 h before SAH operation. The needle remained in place for 5 min and was then withdrawn slowly. The c-Abl mixed sequences of the sense strands were as follows:

1: 5’-CGGCAGCCUAAAUGAAGAUtt-3’,

2: 5’-CCUAUGGCAUGUCACCUUAtt-3’,

3: 5’-GGUUUAUGAGCUGCUGGAAtt-3’.

Scramble siRNA sequence:5’-UUCUCCGAACGUGUCACGUtt-3’.

LRP-1-specific siRNA mixed sequences were as follows:

1: 5’- UUCUAUUCUUAUCCUUUCC tt-3’,

2: 5’- UUACUUCUUGUCCUCGCCC tt-3’,

3: 5’- AAUCUUUGCAUGUCUUGCC tt-3’.

Scramble siRNA sequence:5’-ACUGCUUAGCGUCAAUUCGtt-3’.

Neurological scoring

The modified Garcia scale was used to blindly assess neurological deficits in animals 24 h after SAH as previously reported[19].The assessment system consisted of six subtests to evaluate the following: spontaneous activity, spontaneous movement of the 4 limbs, forepaw outstretching, climbing, body proprioception and the response to whisker stimulation. Each subtest was scored from 0-3 or 1-3, and the total score (from 3 to 18) reflected neurological function (table 1). The lower a score was, the worse the neurological function was.

SAH grade assessment

SAH severity was quantified with an SAH grading scale based on the amount of bleeding as previously described[20].The basal cistern of the brain was photographed and divided into six predetermined segments (Fig 1B), and each segment was scored from 0 to 3, Grade 0:no subarachnoid blood; Grade 1: minimal subarachnoid blood; Grade 2: moderate blood clot with recognizable arteries; Grade3: blood clot obliterating all arteries. The total SAH grade was equal to the sum of all segment scores (maximum SAH grade=18). Rats in which the SAH grade was <8 and those for which SAH coexisted with subdural or epidural hemorrhage were excluded.

Western blot analysis

Western blotting was conducted as previously reported[18,21]. Briefly, the cerebral cortex from the left hemorrhagic site was collected as a sample 24 h after SAH. After anesthesia with pentobarbital（40 mg/kg）, the rats were decapitated and then perfused with glacial PBS transcardially. The soft tissue and skull were separated to obtain the brain tissue. Fresh brain tissue was carefully removed from the basal cortex. The brain tissues were immediately frozen in liquid nitrogen and stored at 80 °C until used (all operations were performed on ice). Tissues were weighed and homogenized. RIPA lysis buffer and protease inhibitor (PMSF, NaF, etc.) were added to the homogenates, which were lysed on ice for 40-60 min and shaken once every 5-8 min. The samples were centrifuged to collect the supernatants. The protein concentration was detected with a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, USA). Samples containing the same amount of the target protein (30 μg) were separated by SDS-PAGE, and the proteins were then transferred to a PVDF membrane. The membrane was blocked with 5% nonfat dry milk for 2 h and incubated overnight at 4°C in the presence of the following primary antibodies: anti β-actin (Abcam, cat# ab6276, 1:1000), anti p-Akt (Abcam CST, cat# ab81283, 1:1000), anti p-GSK3β (Cell Signaling Technology, cat# 8566, 1:2000), anti-CC3 (Cell Signaling Technology, cat# 9661, 1:1000), anti-c-Abl (Abcam, cat# ab47315, 1:1000) and anti LRP-1 (Abcam, cat# ab92544, 1:1000).After washing with TBST buffer, the membrane was incubated with appropriate secondary antibodies (1:2000) at room temperature for 1 h. An ECL Plus chemiluminescence kit (Amersham Bioscience, Arlington Heights, IL)

was used to display the protein bands according to the manufacturer's instructions. ImageJ software (NIH) was used to quantify band densities.

TUNEL staining and NeuN double immunofluorescence labeling

After anesthesia, the rats were perfused transcardially with 4℃ PBS followed by 4% paraformaldehyde. Then, the brain tissue was taken out immediately and fixed in 4% paraformaldehyde overnight. Sucrose solution was dehydrated in gradient, after rinsed, the brain tissue was quickly frozen on the machine. Finally, the continuous coronal frozen section was made. TUNEL and staining for NeuN, a neuronal marker, were used together to detect neuronal apoptosis. Briefly, frozen sections were rewarmed at room temperature for 20 min, blocked with 5% sheep serum(Equitech-Bio,SS-0100) for 1 h and then incubated with anti-NeuN primary antibody (Cell Signaling Technology, cat# 12943, 1:200 dilution). A TUNEL kit (Roche, cat# 11684795910) was used to label apoptotic cells after the use of anti-NeuN secondary antibody (Alexa Fluor® Plus 594-conjugated). The sections were incubated with 50 μL of TUNEL reaction mixture (enzyme solution:labeling solution = 1:9), incubated at 37°C in the dark for 60 min, and washed 3 times with PBS for 5 min each, then the sections were sealed and observed by fluorescence microscopy (Olympus, Tokyo, Japan). TUNEL-positive cells in five different fields were counted. The results are expressed as cells/mm2, and the apoptotic ratio was calculated as the number of apoptotic cells/the total cell number × 100%.

Statistical analysis

All data are expressed as the mean ± SD. Statistical significance was analyzed using one-way ANOVA followed by Tukey's multiple comparison test. A probability value of $p < 0.05$ indicated statistical significance. SPSS 13.0 software for Windows was used to perform all statistical analyses.

Results

Mortality, SAH grade and Survival Curves

No animals from the sham group died. The mortality rates of the SAH were significantly increased and inhibiting c-Abl with specific siRNA reduced the mortality. The mortality of LY294002 group was higher than that of SAH+c-Abl siRNA+vehicle group.Similarly, the mortality rates of SAH+c-Abl siRNA+RAP (or LRP-1 siRNA) were higher than that of SAH+c-Abl siRNA+vehicle group (Fig 1A).Fig 1B shows representative images of the brains of rats from the sham and SAH groups. There was no significant difference in SAH severity between the SAH and SAH + c-Abl siRNA groups($p > 0.05$, Fig 1C). There were no significance between the SAH + Scramble siRNA and SAH + c-Abl siRNA groups by Log-rank (Mantel-Cox) test (Fig 1D).

Inhibition of c-Abl decreased SAH-induced neuronal apoptosis and improved neurologic deficits

TUNEL and NeuN double staining were performed to determine neuronal apoptosis. As shown in Fig 2, the number of double-positive cells following TUNEL and NeuN staining was significantly higher in the

SAH group than in the sham group ($p < 0.05$; Fig 2A, B). However, c-Abl inhibition using specific siRNA significantly decreased neuronal apoptosis compared to that in the SAH and SAH+scramble siRNA groups ($p < 0.05$,Fig 2A, B).Neurological deficits were assessed 24 h after SAH. Compared with the sham group, the nerve function scores in the SAH group were decreased significantly. The inhibition of c-Abl improved neurologic deficits($p<0.05$ vs the SAH group, Fig 2C).

Inhibition of c-Abl promoted the phosphorylation of Akt and GSK3β after SAH

Western blotting and was conducted to verify the levels of c-Abl,the phosphorylation of Akt ,GSK3β and the apoptosis-related protein CC-3.The results showed that c-Abl expression increased after SAH, and was most significant 24 and 48 h after SAH ($p < 0.05$,Fig 3A, B).The phosphorylation of Akt and its downstream protein GSK3β were increased in the SAH group compared to the sham group in our study, which was consistent with the results of a previous study[12]. When c-Abl expression was blocked by specific siRNA, the phosphorylation of Akt and GSK3β was notably increased.Western blotting also showed that the inhibition of c-Abl downregulated SAH-induced CC-3 expression($p < 0.05$,Fig 3C, D, E,F,G).

LY294002 abrogated the effect of c-Abl inhibition on neuronal apoptosis, neurological scores and the CC-3protein level

LY294002, a highly selective inhibitor of the PI3K/Akt signaling pathway, was used to elucidate the role of the Akt/GSK3β signaling pathway in the neuroprotective mechanism induced by inhibiting c-Abl. The inhibition of c-Abl significantly increased the activity of Akt/GSK3β(Fig. 3) and decreased the number of TUNEL-NeuN double-positive cells after SAH, but LY294002 blocked this beneficial effect ($p<0.05$, Fig 4A,B).The improvement in neurological score in response to c-Abl inhibition was markedly abrogated by LY294002 ($p<0.05$, Fig 4C). LY294002 suppressed the phosphorylation of Akt and GSK3β in response to c-Abl siRNA treatment ($p< 0.05$, Fig 5A, C, D),while increasing the expression of CC-3 without influencing c-Abl expression ($p> 0.05$, Fig 5A, B,E).

Downregulation of LRP-1 suppressed the increases in p-Akt and p-GSK3β induced by c-Abl inhibition after SAH

To investigate the effects of LRP-1 downregulation on the activity of Akt/GSK3β induced by inhibiting c-Abl after SAH, LRP-1 was downregulated with a specific siRNA and the inhibitor RAP, and Akt/GSK3β activity was detected by Western blotting. The results showed that LRP-1 levels were significantly decreased after SAH in the SAH group in comparison with the sham group and that the specific siRNA and inhibitor (RAP) further reduced LRP-1 levels ($p< 0.05$,Fig 6A,B,C). In the control groups (SAH+vehicle and SAH+ scramble siRNA groups), we observed increased Akt and GSK3β phosphorylation in response to c-Abl siRNA treatment. This effect was not observed in groups in which LRP-1 downregulation was induced by systemic siRNA delivery and RAP ($p< 0.05$,Fig 6D-H).

Discussion

Our results in an SAH rat model demonstrated for the first time that c-Abl plays a proapoptotic role and confirmed that its mechanism may be related to the LRP-1-dependent Akt/GSK3β signaling pathway (Fig 7). In this study, we observed that the suppression of c-Abl by siRNA significantly improved neurological deficits, downregulated CC-3 and decreased neuronal apoptosis in response to SAH. These results showed that c-Abl was involved in apoptotic mechanisms in EBI after SAH and that inhibiting c-Abl can ameliorate poor outcomes. In addition, Western blotting results suggested that the suppression of c-Abl further enhanced the expression of p-Akt and p-GSK3β induced by EBI following SAH. We used LY294002, a selective inhibitor of PI3K/Akt, to treat SAH rats that had been administered c-Abl siRNA. LY294002 abolished the beneficial effects of c-Abl blockade on neurological outcomes and apoptosis. LRP-1 was decreased after SAH, and LRP-1 expression was downregulated by RAP and LRP-1 siRNA in the presence of c-Abl siRNA, resulting in a significant decrease in Akt/GSK3β activation. These results suggest that c-Abl contributes to SAH-mediated neuronal apoptosis via suppressing the LRP-1-dependent Akt/GSK3β pathway.

c-Abl is an src-associated nonreceptor tyrosine kinase that is widely expressed in the nuclei and cytoplasm of nerve cells. Nuclear c-Abl mainly regulates the cell cycle, determines cell fate and participates in the development and morphogenesis of neurons[22-24].c-Abl has little known function in fully differentiated neurons. c-Abl has been reported to be active in diseases such as prion disease, cerebral ischemia, Parkinson's disease and Alzheimer's disease[9-11,25].Suppression of the c-Abl/p73 pathway was reported to inhibit neuronal apoptosis and improve neuronal dysfunction in an Niemann-Pick Type C disease mouse model[26],but c-Abl overexpression induced neuronal death and increased neuronal inflammation in the mouse forebrain[27].Oxidative stress activates c-Abl in neurons, which then activates p53 or p73 to initiate neuronal apoptosis[28,29].Reactive oxygen free radicals are one of the main mediators of SAH pathology. Accumulating evidence indicates that reactive oxygen species (ROS) production and oxidative stress begin to emerge early after SAH[30].

Despite these advances, whether c-Abl is involved in the apoptotic process in response to SAH has not been reported. In the present study, we determined that c-Abl expression and neuronal apoptosis were notably increased 24 h after SAH and that c-Abl inhibition decreased neuronal apoptosis and improved neurological deficits. Our results showed that c-Abl also had a proapoptotic effect in the pathogenesis of SAH.

Akt, also called protein kinase B, plays an essential role in a variety of important cellular processes, including cell survival, proliferation, and apoptosis[31].GSK3β, a downstream protein of Akt, is a survival pathway protein that mediates cell survival and apoptosis in numerous pathological states[12].It has been reported that GSK3β activity may be regulated by its phosphorylation at tyrosine-216 (irritant) and serine-9 (inhibitory)[32,33].Activated Akt (serine-473) phosphorylates GSK3β on serine-9 to inhibit its activity and reduce apoptosis[34].Accumulated data in an SAH experimental model suggest that the Akt pathway is involved in the biological process of neuron survival. The phosphorylation of Akt and GSK3β has been correlated with EBI after SAH[12].There have been some hypotheses on the downstream mechanisms of the Akt/GSK3β pathway, although these mechanisms are not yet understood. For

instance, the Akt pathway inhibits activity of the proapoptotic kinase GSK3β, which inhibits neuronal apoptosis dependent on the apoptosis-related protein caspase pathway or the mitochondrial pathway by regulating Bcl2/Bax levels[35].Further study of the effectors downstream of Akt/GSK3β is necessary to elucidate the mechanism of acute brain injury after SAH.

Therefore, we determined levels of the c-Abl and p-Akt/p-GSK3β proteins in SAH rats. The c-Abl and p-Akt/p-GSK3β protein levels were markedly increased in the SAH group, which was consistent with a previous report[12].Nevertheless, c-Abl siRNA administration significantly reduced c-Abl expression but increased Akt/GSK3β phosphorylation. These results seem contradictory because of the interaction between the apoptotic and survival mechanisms after SAH. Accordingly, we make two assumptions. First, medium is necessary for c-Abl to regulate the activity of the downstream products of Akt/GSK3β after SAH. Second, other mechanisms regulate Akt/GSK3β activity after SAH. The present results showed that blockade of the Akt/GSK3β pathway with LY294002 abolished the reduction in neuronal apoptosis and improvement in neurological score induced by c-Abl inhibition. Thus, we deduce that c-Abl exerts a proapoptotic effect on neurons by inhibiting the Akt/GSK3β survival pathway after SAH. However, the detailed mechanism of the interaction of c-Abl with Akt/GSK3β requires further research.

LRP-1, a multifunctional multiligand receptor involved in the regulation of endocytosis and many cellular processes[13],is abundantly expressed in cortical and hippocampal neurons in the brain[14].LRP-1 has a variety of biological neuronal functions closely related to the maintenance of synapses, metabolism of lipoproteins, and clearance of amyloid-β (Aβ) in the brain and participate in various mechanisms of neuron survival[14].Previous studies have shown that LRP-1 regulates the downstream insulin receptor and Akt pathway to inhibit neuronal apoptosis in the pathological state of Alzheimer's disease[14].According to previous data, many important cytoplasmic adaptors in signal transduction can bind the tail of LRP-1. For example, an experiment in yeast confirmed that Dab1 specifically binds the cytoplasmic tail of LRP-1 at the second NPXY motif[36,37].Activation of the Akt pathway by α2M through LRP-1 has been well characterized in neurons. LRP-1 was found to bind α2M in Schwann and PC12 cells, promoting the binding of LRP-1 to Dab1 and then activating the Akt pathway[38].However, c-Abl inhibition by imatinib increased LRP-1-dependent ERK and Akt activation and contributed to pancreatic β cell function and survival. It was reported that the LRP-1 was a regulator between c-Abl and Akt, LRP-1 was indispensable for the effects of c-Abl in inhibiting Akt expression[16]. We administrated RAP 1 hour and specific siRNA 24 hour before SAH induction to inhibit LRP-1 and further disturbed the interaction between c-Abl and Akt/GSK3β.The objective of this experiment was further illustrated the role of Akt/GSK3β in the effects of c-Abl on apoptosis. Our work provides the first evidence suggesting a link between c-Abl function and LRP-1-dependent Akt/GSK3β signaling in SAH. In the present work, we observed increased Akt and GSK3β phosphorylation in response to c-Abl siRNA after SAH, but LRP-1 downregulation with an siRNA or inhibitor suppressed the increase in p-Akt and p-GSK3β induced by c-Abl siRNA. This finding indicates that LRP-1 is necessary for c-Abl-mediated regulation of Akt/GSK3β activity in SAH.

Conclusions

These results associate SAH-induced c-Abl overexpression with neuronal apoptosis in EBI and suggest that inhibiting c-Abl improves neurological deficits. In addition, the proapoptotic effect of c-Abl following SAH might be mediated via the LRP-1-dependent Akt/GSK3β signaling pathway.

Declarations

Acknowledgement

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest

All authors declare that there is no conflict of interest in this study.

Research involving Human Participants and/or Animals

All procedures involving animals were in accordance with the ethical standards of of the First Affiliated Hospital of Harbin Medical University.

Informed consent

This research group uses animals for experimental research and passes the ethical review without involving informed consent.

References

1. Feigin VL, Lawes CM, Bennett DA, Barker-Collo SL, Parag V (2009) Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review. Lancet Neurol 8 (4):355-369. doi:10.1016/s1474-4422(09)70025-0
2. Al-Khindi T, Macdonald RL, Schweizer TA (2010) Cognitive and functional outcome after aneurysmal subarachnoid hemorrhage. Stroke 41 (8):e519-536. doi:10.1161/strokeaha.110.581975
3. Kassell NF, Sasaki T, Colohan AR, Nazar G (1985) Cerebral vasospasm following aneurysmal subarachnoid hemorrhage. Stroke 16 (4):562-572
4. Macdonald RL, Kassell NF, Mayer S, Ruefenacht D, Schmiedek P, Weidauer S, Frey A, Roux S, Pasqualin A, Investigators C- (2008) Clazosentan to overcome neurological ischemia and infarction occurring after subarachnoid hemorrhage (CONSCIOUS-1): randomized, double-blind, placebo-controlled phase 2 dose-finding trial. Stroke 39 (11):3015-3021. doi:10.1161/STROKEAHA.108.519942

5. Kusaka G, Ishikawa M, Nanda A, Granger DN, Zhang JH (2004) Signaling pathways for early brain injury after subarachnoid hemorrhage. J Cereb Blood Flow Metab 24 (8):916-925. doi:10.1097/01.wcb.0000125886.48838.7e
6. Fujii M, Yan J, Rolland WB, Soejima Y, Caner B, Zhang JH (2013) Early brain injury, an evolving frontier in subarachnoid hemorrhage research. Transl Stroke Res 4 (4):432-446. doi:10.1007/s12975-013-0257-2
7. Peng Y, He P, Fan L, Xu H, Li J, Chen T, Ruan W, Dou Z, Chen G (2018) Neuroprotective Effects of Magnesium Lithospermate B against Subarachnoid Hemorrhage in Rats. Am J Chin Med 46 (6):1225-1241. doi:10.1142/S0192415X18500647
8. Nau R, Haase S, Bunkowski S, Bruck W (2002) Neuronal apoptosis in the dentate gyrus in humans with subarachnoid hemorrhage and cerebral hypoxia. Brain Pathol 12 (3):329-336
9. Schlatterer SD, Acker CM, Davies P (2011) c-Abl in neurodegenerative disease. J Mol Neurosci 45 (3):445-452. doi:10.1007/s12031-011-9588-1
10. Brahmachari S, Ge P, Lee SH, Kim D, Karuppagounder SS, Kumar M, Mao X, Shin JH, Lee Y, Pletnikova O, Troncoso JC, Dawson VL, Dawson TM, Ko HS (2016) Activation of tyrosine kinase c-Abl contributes to alpha-synuclein-induced neurodegeneration. J Clin Invest 126 (8):2970-2988. doi:10.1172/jci85456
11. Wu R, Chen H, Ma J, He Q, Huang Q, Liu Q, Li M, Yuan Z (2016) c-Abl-p38alpha signaling plays an important role in MPTP-induced neuronal death. Cell Death Differ 23 (3):542-552. doi:10.1038/cdd.2015.135
12. Endo H, Nito C, Kamada H, Yu F, Chan PH (2006) Akt/GSK3beta survival signaling is involved in acute brain injury after subarachnoid hemorrhage in rats. Stroke 37 (8):2140-2146. doi:10.1161/01.STR.0000229888.55078.72
13. Lin L, Hu K (2014) LRP-1: functions, signaling and implications in kidney and other diseases. Int J Mol Sci 15 (12):22887-22901. doi:10.3390/ijms151222887
14. Fuentealba RA, Liu Q, Kanekiyo T, Zhang J, Bu G (2009) Low density lipoprotein receptor-related protein 1 promotes anti-apoptotic signaling in neurons by activating Akt survival pathway. J Biol Chem 284 (49):34045-34053. doi:10.1074/jbc.M109.021030
15. Lok KZ, Manzanero S, Arumugam TV (2016) Neuronal low-density lipoprotein receptor-related protein 1 (LRP1) enhances the anti-apoptotic effect of intravenous immunoglobulin (IVIg) in ischemic stroke. Brain Res 1644:192-202. doi:10.1016/j.brainres.2016.05.023
16. Fred RG, Boddeti SK, Lundberg M, Welsh N (2015) Imatinib mesylate stimulates low-density lipoprotein receptor-related protein 1-mediated ERK phosphorylation in insulin-producing cells. Clin Sci (Lond) 128 (1):17-28. doi:10.1042/CS20130560
17. Park S, Yamaguchi M, Zhou C, Calvert JW, Tang J, Zhang JH (2004) Neurovascular protection reduces early brain injury after subarachnoid hemorrhage. Stroke 35 (10):2412-2417. doi:10.1161/01.STR.0000141162.29864.e9

18. Suzuki H, Ayer R, Sugawara T, Chen W, Sozen T, Hasegawa Y, Kanamaru K, Zhang JH (2010) Protective effects of recombinant osteopontin on early brain injury after subarachnoid hemorrhage in rats. Crit Care Med 38 (2):612-618. doi:10.1097/CCM.0b013e3181c027ae
19. Yin C, Huang GF, Sun XC, Guo Z, Zhang JH (2016) Tozasertib attenuates neuronal apoptosis via DLK/JIP3/MA2K7/JNK pathway in early brain injury after SAH in rats. Neuropharmacology 108:316-323. doi:10.1016/j.neuropharm.2016.04.013
20. Sugawara T, Ayer R, Jadhav V, Zhang JH (2008) A new grading system evaluating bleeding scale in filament perforation subarachnoid hemorrhage rat model. J Neurosci Methods 167 (2):327-334. doi:10.1016/j.jneumeth.2007.08.004
21. Cheng G, Chunlei W, Pei W, Zhen L, Xiangzhen L (2010) Simvastatin activates Akt/glycogen synthase kinase-3beta signal and inhibits caspase-3 activation after experimental subarachnoid hemorrhage. Vascul Pharmacol 52 (1-2):77-83. doi:10.1016/j.vph.2009.12.001
22. Leonberg AK, Chai YC (2007) The functional role of cysteine residues for c-Abl kinase activity. Mol Cell Biochem 304 (1-2):207-212. doi:10.1007/s11010-007-9501-y
23. Nagar B, Hantschel O, Young MA, Scheffzek K, Veach D, Bornmann W, Clarkson B, Superti-Furga G, Kuriyan J (2003) Structural basis for the autoinhibition of c-Abl tyrosine kinase. Cell 112 (6):859-871
24. Smith JM, Mayer BJ (2002) Abl: mechanisms of regulation and activation. Front Biosci 7:d31-42
25. Sun R, Song Y, Li S, Ma Z, Deng X, Fu Q, Qu R, Ma S (2018) Levo-tetrahydropalmatine Attenuates Neuron Apoptosis Induced by Cerebral Ischemia-Reperfusion Injury: Involvement of c-Abl Activation. J Mol Neurosci 65 (3):391-399. doi:10.1007/s12031-018-1063-9
26. Alvarez AR, Klein A, Castro J, Cancino GI, Amigo J, Mosqueira M, Vargas LM, Yevenes LF, Bronfman FC, Zanlungo S (2008) Imatinib therapy blocks cerebellar apoptosis and improves neurological symptoms in a mouse model of Niemann-Pick type C disease. FASEB J 22 (10):3617-3627. doi:10.1096/fj.07-102715
27. Schlatterer SD, Tremblay MA, Acker CM, Davies P (2011) Neuronal c-Abl overexpression leads to neuronal loss and neuroinflammation in the mouse forebrain. J Alzheimers Dis 25 (1):119-133. doi:10.3233/JAD-2011-102025
28. Klein A, Maldonado C, Vargas LM, Gonzalez M, Robledo F, Perez de Arce K, Munoz FJ, Hetz C, Alvarez AR, Zanlungo S (2011) Oxidative stress activates the c-Abl/p73 proapoptotic pathway in Niemann-Pick type C neurons. Neurobiol Dis 41 (1):209-218. doi:10.1016/j.nbd.2010.09.008
29. Lee JH, Jeong MW, Kim W, Choi YH, Kim KT (2008) Cooperative roles of c-Abl and Cdk5 in regulation of p53 in response to oxidative stress. J Biol Chem 283 (28):19826-19835. doi:10.1074/jbc.M706201200
30. Chen S, Feng H, Sherchan P, Klebe D, Zhao G, Sun X, Zhang J, Tang J, Zhang JH (2014) Controversies and evolving new mechanisms in subarachnoid hemorrhage. Prog Neurobiol 115:64-91. doi:10.1016/j.pneurobio.2013.09.002
31. Manning BD, Cantley LC (2007) AKT/PKB signaling: navigating downstream. Cell 129 (7):1261-1274. doi:10.1016/j.cell.2007.06.009

32. Bhat RV, Shanley J, Correll MP, Fieles WE, Keith RA, Scott CW, Lee CM (2000) Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3beta in cellular and animal models of neuronal degeneration. Proc Natl Acad Sci U S A 97 (20):11074-11079. doi:10.1073/pnas.190297597
33. Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65 (4):391-426
34. Hetman M, Cavanaugh JE, Kimelman D, Xia Z (2000) Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. J Neurosci 20 (7):2567-2574
35. Si H, Zhang Y, Song Y, Li L (2018) Overexpression of adrenomedullin protects mesenchymal stem cells against hypoxia and serum deprivationinduced apoptosis via the Akt/GSK3beta and Bcl2 signaling pathways. Int J Mol Med 41 (6):3342-3352. doi:10.3892/ijmm.2018.3533
36. Howell BW, Lanier LM, Frank R, Gertler FB, Cooper JA (1999) The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. Mol Cell Biol 19 (7):5179-5188. doi:10.1128/mcb.19.7.5179
37. Trommsdorff M, Borg JP, Margolis B, Herz J (1998) Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. J Biol Chem 273 (50):33556-33560. doi:10.1074/jbc.273.50.33556
38. Mantuano E, Mukandala G, Li X, Campana WM, Gonias SL (2008) Molecular dissection of the human alpha2-macroglobulin subunit reveals domains with antagonistic activities in cell signaling. J Biol Chem 283 (29):19904-19911. doi:10.1074/jbc.M801762200

Tables

Table 1 modified Garcia score

	scores			
	0	1	2	3
spontaneous activity	No movement	Barely moves	Moves but not approach at least three sides of cage	Moves and approaches at least three sides of cage
spontaneous movement of the 4 limbs	No movement	contralateral side: slight movement	contralateral side :Moves slowly	Both sides: Moves symmetrically
forepaw outstretching	contralateral side: no	contralateral side: slight	contralateral side: moves and outstretch less than ipsilateral side	Symmetrical outstretch
climbing	–	Fails to climb	contralateral side is weak	normal
body proprioception	—	No response on contralateral side	Weak response on contralateral side	Symmetrical response
the response to whisker stimulation	–	No response on contralateral side	Weak response on contralateral side	Symmetrical response

Figures

A

Groups	Mortality	Exclusion
1-1		
(1)Sham	0 (0 of 6)	0
(2)SAH 24h	25%(2 of 8)	0
(3)SAH48h	33.33%(3 of 9)	0
(4)SAH72h	14.29% (1 of 7)	0
1-2		
(1)Sham	0 (0 of 12)	0
(2)SAH	29.41%(5 of 17)	1
(3)SAH+scramble siRNA	33.33%(6 of 18)	0
(4)SAH+c-Abl siRNA	14.29% (2 of 14)	0
2		
(1)SAH+scramble siRNA+vehicle	29.41%(5 of 17)	1
(2)SAH+c-Abl siRNA+vehicle	14.29%(2 of 14)	0
(3)SAH+c-Abl siRNA+LY294002	20%(3 of 15)	0
3		
(1)sham	0 (0 of 6)	0
(2)SAH+vehicle/scramble siRNA	14.29%(1 of 7)/14.29%(1 of 7)	1
(3)SAH+RAP/LRP1 siRNA	25%(2 of 8)/25%(2 of 8)	1
4		
(1)SAH+c-Abl siRNA+vehicle/scramble siRNA	14.29%(1 of 7)/%14.29(1 of 7)	0
(2)SAH+c-Abl siRNA+RAP/LRP1 siRNA	25%(2 of 8)/25%(2 of 8)	0
Total		4

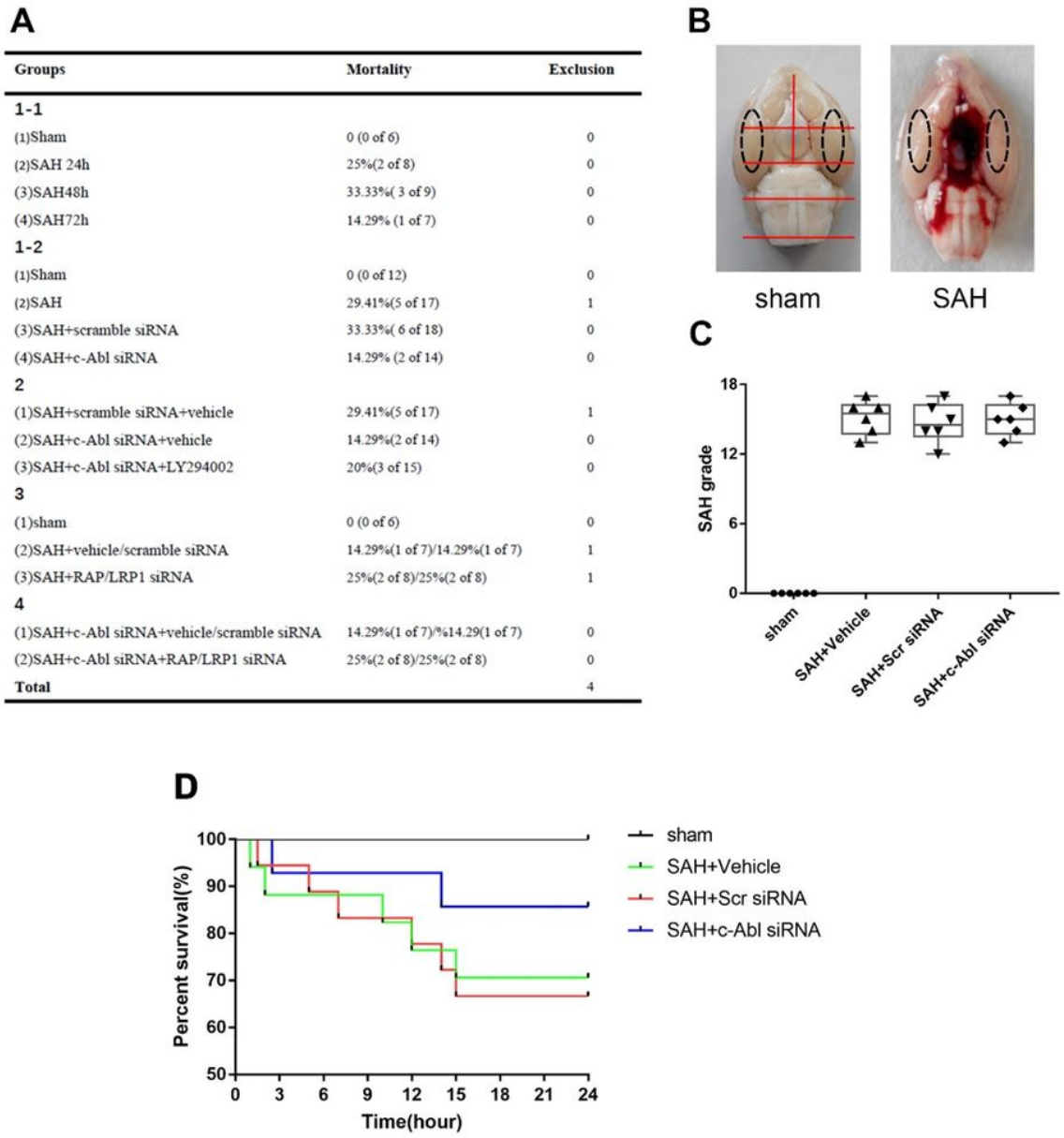

Figure 1

Mortality, SAH grade and survival curves 24h of sham and experimental groups after SAH.A Mortality of every groups. B Typical pictures of brains from sham and 24 h after SAH. The elliptic regions were the sampling area of Western blot and staining, in which the coronal section of the specimen used for staining was adopted. C c-Abl inhibition had no effect on the SAH grade after SAH. D Curves of survival rate from every group within 24 h after SAH. There was no significant difference between SAH + Scramble siRNA and SAH + c-Abl siRNA groups by log rank (mantel Cox) test. n=12 per group.

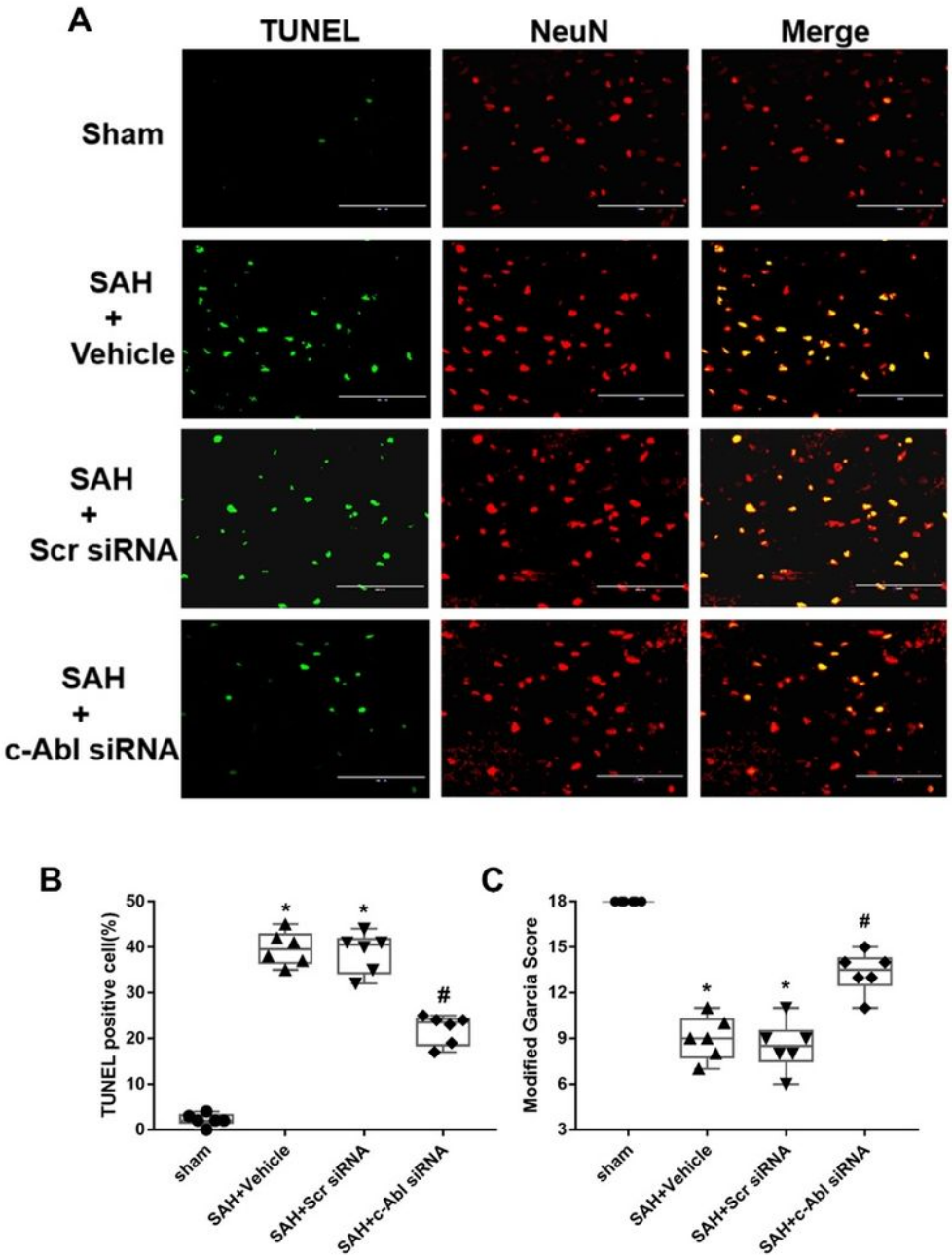

Figure 2

Inhibiting c-Abl reduced TUNEL positive neurons and improved neurologic deficits 24h after SAH. A Representative immunofluorescence staining showed TUNEL and NeuN double-stained cells (TUNEL+neurons) from every group. B Inhibiting c-Abl significantly decreased the number of TUNEL-positive neurons after SAH.C The inhibition of c-Abl improved neurologic deficits. n=6 per group. *p < 0.05 versus sham, #p < 0.05 versus SAH+Vehicle/SAH+scramble siRNA.(Scale bar, 100 μm)

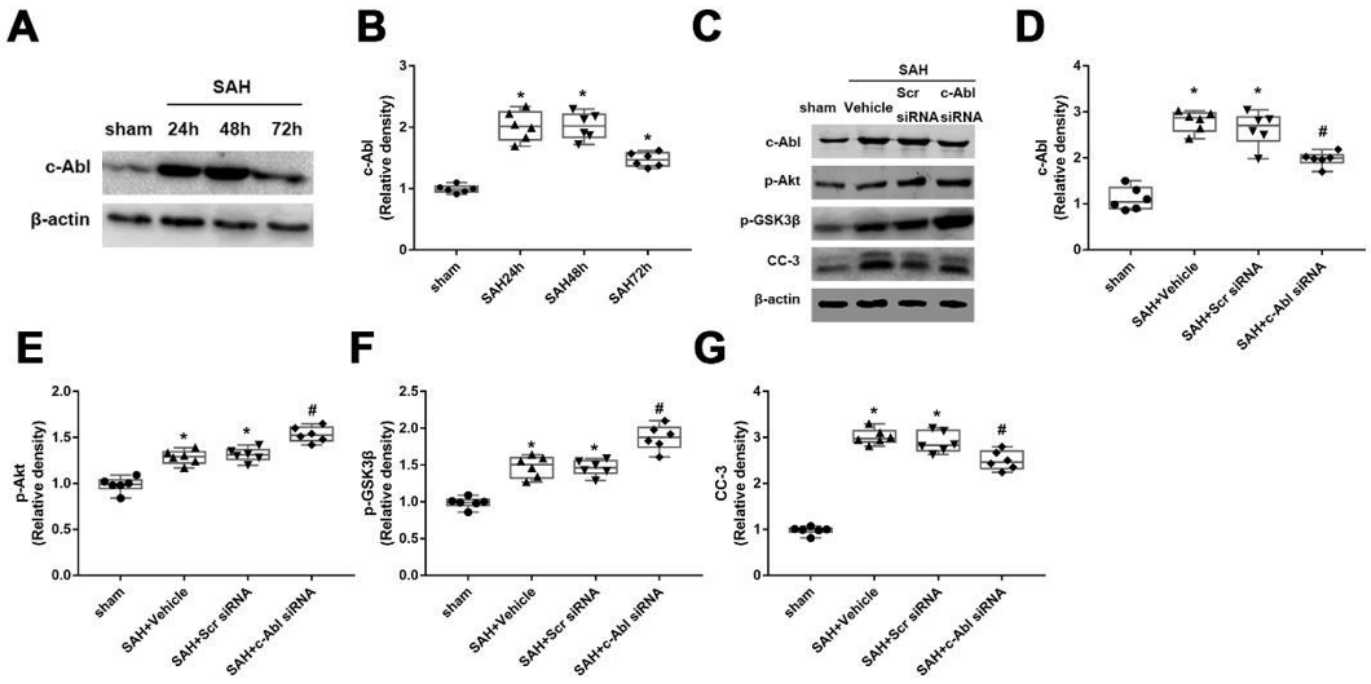

Figure 3

Effects of inhibiting c-Abl on the expression of p-Akt/p-GSK3β/CC-3 in the ipsilateral cortex 24 h after SAH. c-Abl expression increased after SAH, and arrived at the climax at 24 and 48 h. Inhibition of c-Abl promoted the phosphorylation of Akt and GSK3β after SAH,but downregulated CC-3 expression. A, C Representative western blots. B, D, E, F, G Quantitative analysis of c-Abl,p-Akt,p-GSK3β and CC-3. n=6 per group. *p < 0.05 versus sham, #p < 0.05

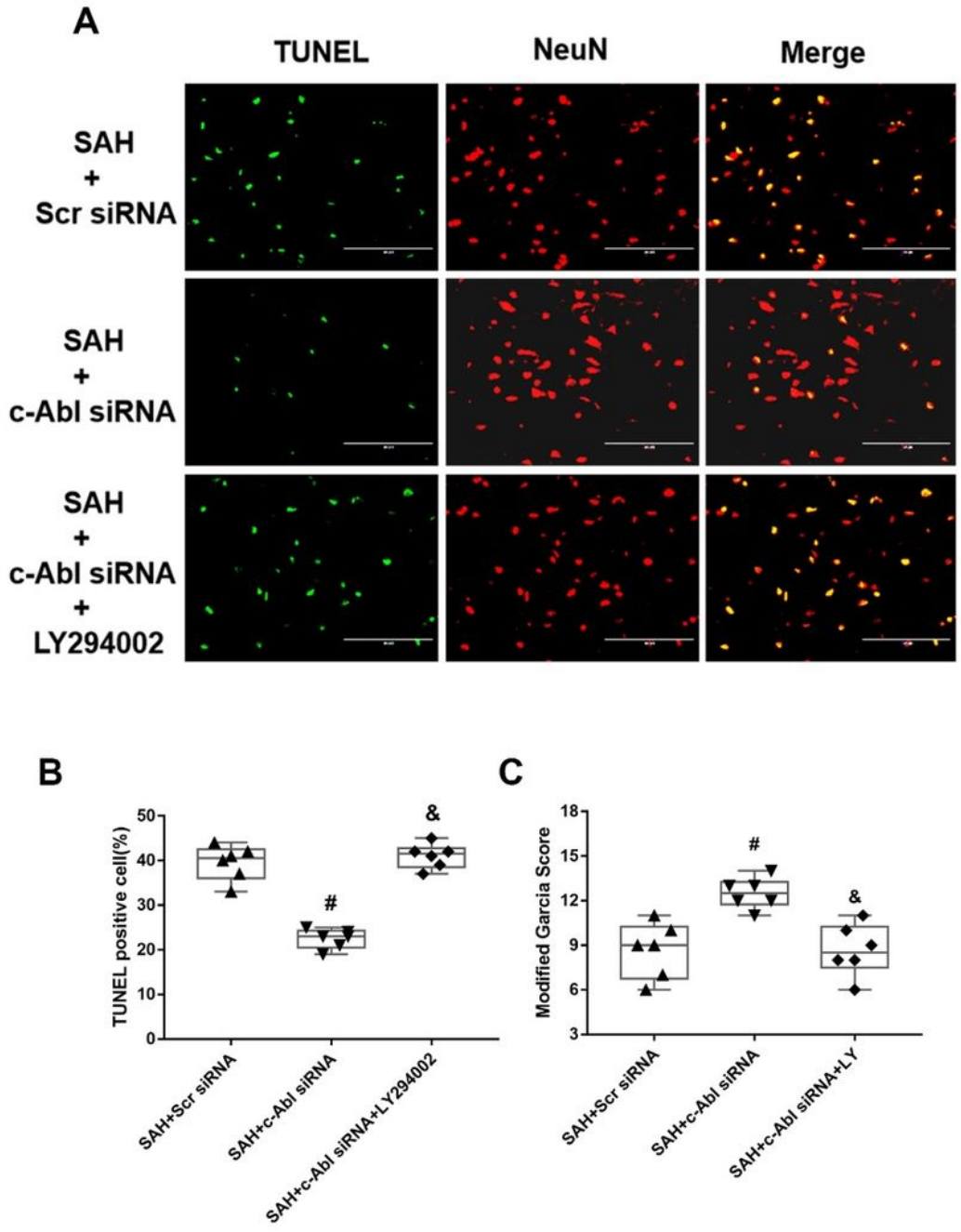

Figure 4

LY294002 abolished the effects of suppressing c-Abl, aggravated neuronal apoptosis and neurological deficits after SAH.A Representative immunofluorescence staining showed TUNEL and NeuN double-stained cells (TUNEL+neurons) from every group. B,C LY294002 abolished the effects of suppressing c-Abl,increased notably the number of TUNEL-positive neurons and aggravated the neurologic function

after SAH. n=6 per group. #p < 0.05 versus SAH+scramble siRNA,&p < 0.05 versus SAH+c-Abl siRNA. (Scale bar, 100 μm)

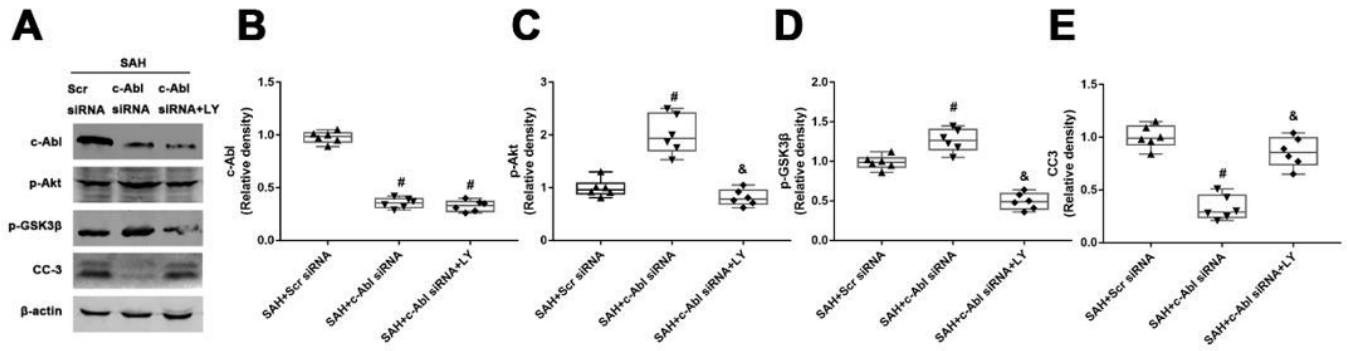

Figure 5

LY294002 abolished the effects of down-regulating c-Abl, reversed the expression of p-Akt/p-GSK3β/CC-3 24 h after SAH. A Representative western blots. B,C,D,E Quantitative analysis of c-Abl, p-Akt,p-GSK3β and CC-3 protein levels of every groups. n=6 per group. #p < 0.05 versus SAH+scramble siRNA,&p < 0.05 versus SAH+c-Abl siRNA.

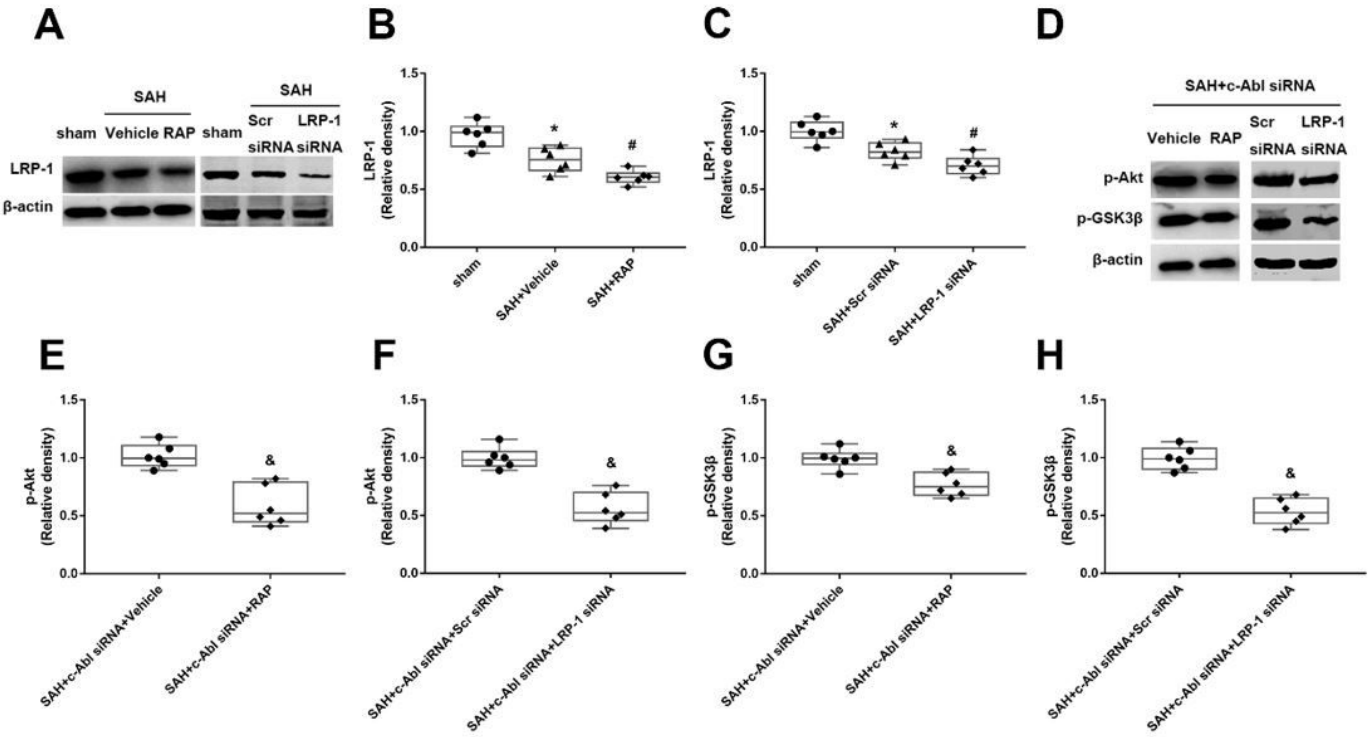

Figure 6

Downregulation of LRP-1 with RAP and siRNA abolished the effects of suppressing c-Abl, reversed the changes in p-Akt, p-GSK3β levels. RAP and LRP-1 siRNA decreased the levels of LRP-1 after SAH. The increases of p-Akt and p-GSK3β expression induced by inhibiting c-Abl were abolished by downregulation of LRP-1 in response to RAP and siRNA respectively. A,D Representative western blots. B, C, E, F, G, H Quantitative analysis of LRP-1,p-Akt and p-GSK3β protein levels. n=6 per group. *p < 0.05 versus sham,#p < 0.05 versus SAH+Vehicle/scramble siRNA,&p < 0.05 versus SAH+c-Abl siRNA+vehicle/scramble siRNA

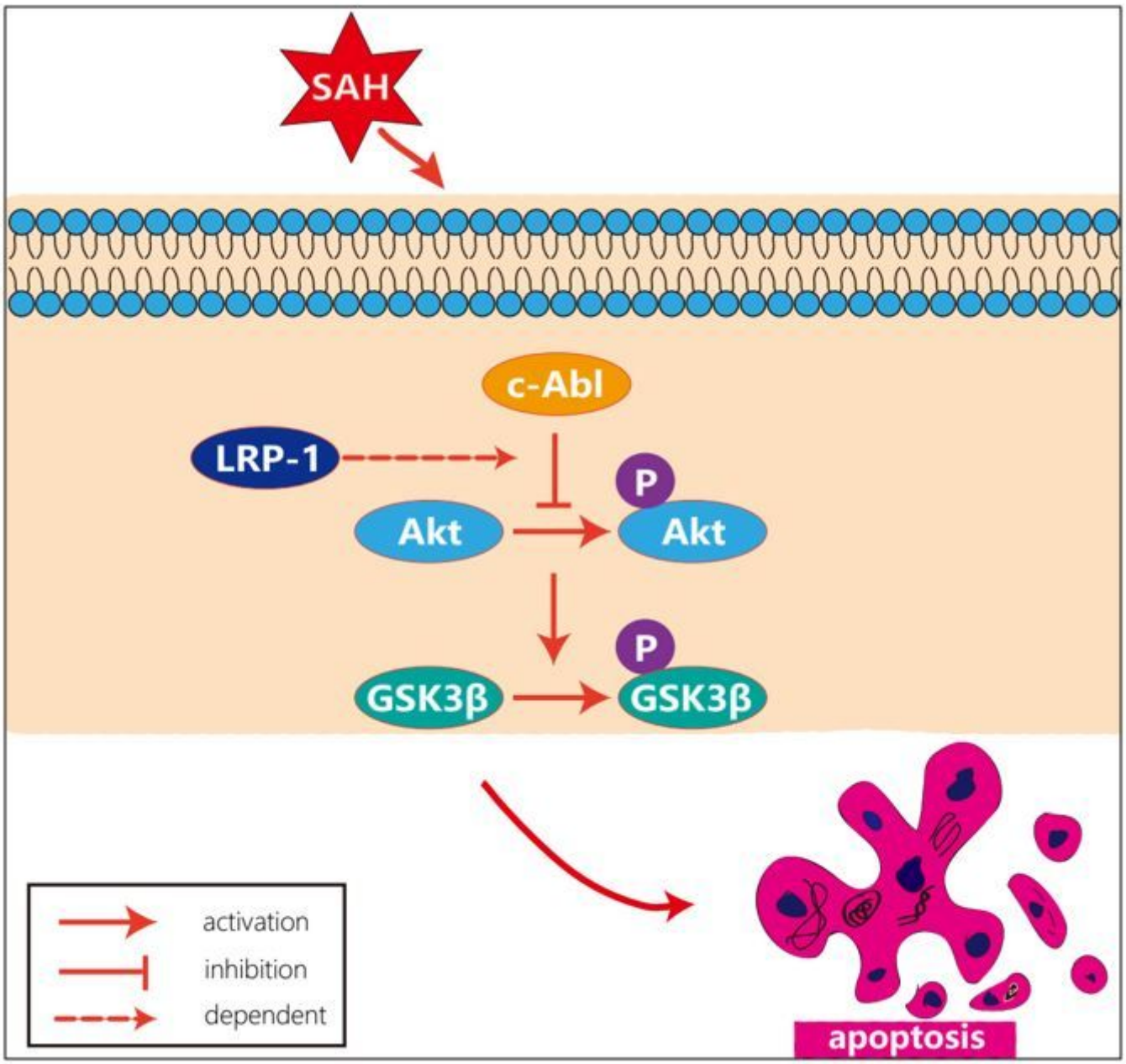

Figure 7

Schematic diagram of c-Abl mediated neuron apoptosis in Subarachnoid Hemorrhage