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Dihydrolipoic acid inhibits lysosomal rupture and NLRP3 through LAMP1/CaMKII/TAK1 pathways after subarachnoid hemorrhage in rat

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Abstract

Background and Purpose—The NLRP3 inflammasome is a crucial component of the inflammatory response in early brain injury (EBI) after subarachnoid hemorrhage (SAH). In this study we investigated a role of dihydrolipoic acid (DHLA) in lysosomal rupture, NLRP3 activation, and determined the underlying pathway.

Methods—SAH was induced by endovascular perforation in male Sprague-Dawley rats. DHLA was administered intraperitoneally 1 hour (h) after SAH. Small interfering ribonucleic acid (siRNA) for lysosome-associated membrane protein-1 (LAMP1) and calcium/calmodulin-dependent protein kinase II α (CaMKIIα) were administered through intracerebroventricular (i.c.v) 48 h before SAH induction. SAH grade evaluation, short and long-term neurological function testing, Western blot and immunofluorescence staining experiments were performed.

Results—DHLA treatment increased the expression of LAMP1 and decreased phosphorylated CaMKIIα (p-CaMKIIα) and NLRP3 inflammasome, thereby alleviating neurological deficits following SAH. LAMP1 siRNA abolished the neuroprotective effects of DHLA and increased the level of p-CaMKIIα, p-TAK1, p-JNK and NLRP3 inflammasome. CaMKIIα siRNA downregulated the expression of p-TAK1, p-JNK and NLRP3 and improved the neurobehavior after SAH.

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Conflict of Interest

There is no conflict of interest.

Conclusions—DHLA treatment improved neurofunction and alleviated inflammation through the LAMP1/CaMKII/TAK1 pathway in EBI after SAH. DHLA may provide a promising treatment to alleviate EBI after SAH.

Keywords

Dihydrolipoic acid; LAMP1; NLRP3; Early brain injury; Subarachnoid hemorrhage

Introduction

Early brain injury (EBI), which occurs within 72 hours (h) after SAH, has recently been considered a major cause of the poor outcome of SAH patients.[1] The underlying mechanisms include a reduction in cerebral blood flow, increased intracranial pressure, oxidative stress, apoptosis, blood-brain barrier disruption and inflammation.[2] Recently, increasing evidence has indicated the role of the NLRP3 inflammasome as a key component of post-SAH inflammatory response.[3, 4] The disruption of the lysosomal membrane and lysosomal rupture leads to the release of cathepsin B/D, which has been shown to induce the activation of the NLRP3 inflammasome.[5] In the SAH rat model, it has also been shown that the lysosomal membrane may be damaged after SAH, which leads to the release of cathepsin B/D and induces apoptosis.[6, 7] Previous studies have shown that lysosomal rupture regulates NLRP3 inflammasome activation through the TAK1/JNK pathway. Additionally, Ca^{2+} ions from lysosomal rupture are an important factor to activate this pathway through calcium/calmodulin-dependent protein kinase II (CaMKII), *in vitro*.[8] However, the mechanisms between lysosomal rupture and NLRP3 activation after SAH are still unclear.

Lysosomal-associated membrane protein-1 (LAMP1) is the most abundant lysosomal membrane protein and is regarded as a marker to evaluate the stability of lysosomes.[9] In addition, studies have shown that the LAMP1 protein not only maintains the structural integrity of the lysosomal membranes, but is also closely associated with cell autophagy and apoptosis.[10, 11] However, the role of LAMP1 contributing to inflammation in EBI after SAH has not been studied.

Dihydrolipoic acid (DHLA), an active form of lipoic acid (LA), is a powerful electron donor, induced by lipoamide dehydrogenase in the cell.[12] It can stabilize the lysosomal membrane, decrease oxidative stress and exert beneficial effects in rat models of various diseases.[6, 13] To date, no study has investigated the anti-inflammation role of DHLA in SAH models. In the present study, we hypothesized that DHLA treatment could inhibit lysosomal rupture and attenuate NLRP3 activation through the LAMP1/CaMKII/TAK1 pathway in early brain injury after SAH.

Material and Methods

The authors declare that all supporting data are available in the article and online supplementary files

Animals and SAH Model

Adult male Sprague–Dawley rats (n=184, 290–330 g) were housed in a room with constant temperature (25 °C), humidity control, a 12/12 hour light/dark cycle and free access to food and water. All the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Loma Linda University, and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

The SAH model was conducted by the modified endovascular perforation method.[14] Briefly, rats were anesthetized and kept on a ventilator during surgery with 3% isoflurane in 65/35% medical air/oxygen. The left external and internal carotid artery were exposed and then a 4.0 monofilament nylon suture was inserted into the left internal carotid artery through the external carotid artery stump until resistance was felt. Then, the suture was advanced 3 mm to perforate the bifurcation of the anterior and middle cerebral artery. Sham rats underwent the same procedures except the perforation. The incision was then closed and rats were housed individually in heated cages following recovery from anesthesia.

Experimental Design (Supplemental Figure I)

Experiment 1—Thirty-eight (38) rats were divided into 6 groups (sham, and SAH after 3, 6, 12, 24 and 72 h, n=6). The additional 2 rats in the SAH (24 h) group were used for immunofluorescence staining. The temporal expression of LAMP1 and p-CaMKIIα was detected by Western blot. Immunofluorescence staining was performed to test the localization of LAMP1 and p-CaMKIIα in the neurons, astrocytes and microglia.

Experiment 2—A total of seventy-two (72) rats were divided into 5 groups: the Sham (n=20 including 6 rats for neurological tests and western blot, 4 rats for immunofluorescence staining, and 10 rats for long-term study), SAH+vehicle (n=20), SAH+DHLA (10 mg/kg, n=6), SAH+DHLA (30 mg/kg, n=20) and SAH+DHLA (90 mg/kg, n=6). Based on neurological tests, 30 mg/kg of DHLA treated group of SAH was chosen for Western blot, immunofluorescence and long-term neurobehavior experiments.

Experiment 3—Thirty (30) Rats were randomly divided into 5 groups: Sham (n=6), SAH +vehicle (n=6), SAH+DHLA (best dosage) (n=6), SAH+DHLA+Scramble siRNA (n=6) and SAH+DHLA+LAMP1 siRNA (n=6). Another 18 rats were randomly divided into 3 groups: Sham (n=6), SAH+Scramble siRNA (n=6) and SAH+CaMKIIα siRNA (n=6). The relevance factors were tested by Western blot.

Drug Administration

DHLA was purchased from Sigma. Before injection, it was diluted in DMSO and PBS. DHLA was administered in the SAH+DHLA group one hour after surgery by intraperitoneal injection (i.p). The SAH+vehicle group received an equal volume of DMSO and PBS.

LAMP1 siRNA (SR508640), CaMKIIα siRNA (SR500835) and scramble siRNA were purchased from Origene Technologies, Inc. (MD, USA). A total of 500 pmol in 5μl was injected intracerebroventricularly (i.c.v) at 48 h pre-surgery as previously described.[15]

Measurement of SAH Grade

The SAH grading score was used to estimate the degree of SAH as previously described.[14] The grading of SAH was performed by a partner who was blind to the experiment. Rats with the SAH grade lower than 9 were excluded from this study.

Assessment of Short Term Neurological Function

The neurological status of all rats was evaluated at 24 h after SAH induction using the previously described modified Garcia scoring system and beam balance test.[14] The assessment of neurological score was performed by a partner who was blind to the experiment.

Assessment of Long-term Neurobehavior

The rotarod test was performed at the first, second and third week post-SAH to assess sensorimotor coordination and balance as previously described.[16] Water maze test was performed at days 21–25 post-SAH as previous study showed.[17]

Western Blot Analysis

Western blot was performed as previously described.[18] Briefly, proteins of the left hemisphere were extracted by homogenizing in RIPA buffer (Santa Cruz Biotechnology, CA, USA). The primary antibodies were from Abcam (MA, USA) used with following dilution: LAMP1 (1:1000, ab24170), CaMKII (1:5000, ab52476), p-CaMKII (1:2000, ab32678), TAK1 (1:1000, ab109536), p-TAK1 (1:1000, ab109404), JNK (1:2000, ab179461), p-JNK (1:2000, ab131499), NLRP3 (1:500, ab214185), and IL-1β (1:500, ab9787). Caspase-1 was from NOVUS (CO, USA, 1:500, NBP1-45433).

Immunofluorescence Staining

Rats were sacrificed at 24 h after SAH induction. A series of 10 μm slices were prepared. Double immunofluorescence staining was performed as previously described.[19] The primary antibodies were LAMP1 (1:200, ab24170), p-CaMKII (1:250, ab32678) and Iba-1 (1:200, ab5076).

Statistical Analysis

Data were presented as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare means of different groups followed by a Tukey multiple-comparisons test. Statistical significance was defined as $P < 0.05$.

Results

Mortality and SAH severity scores

A total of 184 rats were used, 38 rats were sham and 146 rats underwent SAH induction. The mortality of SAH rats was 13.0% (19 of 146) and no rats dying in the sham group. 7 rats were excluded due to low grade SAH. Blood clots were mainly observed around the circle of Willis and ventral brain stem after SAH induction. The average SAH grade score had no statistical difference among all SAH groups (Supplemental Figure II).

Temporal patterns of LAMP1, p-CaMKII were detected in left hemisphere following SAH

Western blot was performed to determine the protein expression of LAMP1, p-CaMKIIα at 3, 6, 12, 24 and 72 h in the left hemisphere of the rat brain after SAH. Results showed that LAMP1 level decreased as early as 3 h after SAH, and reached its lowest level around 24 h ($P < 0.05$, Figure 1A). Additionally, the expression of p-CaMKIIα increased after SAH and peaked at 24 h, and there was notable difference compared to sham animals ($P < 0.05$, Figure 1B). Double immunofluorescence staining was performed to detect localization of the LAMP-1 and p-CaMKIIα in the neurons (NeuN), astrocytes (GFAP) or microglia (Iba-1). We found that LAMP1 was expressed on all type of cells and was mainly colocalized with neuron and microglia in the cortex at 24 h after SAH (Figure 1C). We also found that p-CaMKIIα was mainly colocalized with neuron and microglia (Figure 1D).

DHLA Improved Short and Long-term Neurobehavior

The modified Garcia and beam balance scores were significantly lower in the SAH+vehicle group than those in the sham group ($P < 0.01$, Figure 2A), and the administration of DHLA improved the neurological scores in SAH+DHLA group. The administration of middle dosage (30 mg/kg) significantly improved the neurological scores compared to the SAH +vehicle group ($P < 0.05$, Figure 2A) and seemed to be the most effective dosage. Therefore, we chose this dosage for the long-term and mechanism studies.

In the Rotarod test, the SAH+vehicle group had a significantly shorter latency to fall compared to the sham group both in the 5RPM and 10RPM accelerating velocity tests ($P < 0.01$, Figure 2B). One week after SAH, DHLA treatment improved performance significantly in the 5RPM test ($P < 0.05$, Figure 2B, left panel). Finally, two weeks after SAH, DHLA improved the performance in both the 5RPM and 10RPM tests significantly ($P < 0.05$ in 5RPM, $P < 0.01$ in 10RPM, Figure 2B).

In the water maze test, all the groups performed equally in velocity (Figure 2C, left panel). In the spatial maze test, the SAH+vehicle group traveled a longer distance to find the platform and had a longer escape latency than sham group and it was significantly improved by DHLA treatment on the performance of day 2–4 and block 2–4 in SAH+DHLA group ($P < 0.05$, Figure 2D). In the probe trials, the SAH+vehicle group spent less time in the target quadrant when the platform was removed compared to the sham group. DHLA treatment notably improved the duration spent in the probe quadrant for the SAH+DHLA group ($P < 0.05$, Figure 2C, right panel).

DHLA increased the expression of LAMP1 and inhibited the expression of p-CaMKIIα, NLRP3, cleaved caspase-1, IL-1β

At 24 h after SAH, the expression of LAMP1 was remarkably decreased, whereas p-CaMKIIα, NLRP3, cleaved caspase-1 and IL-1β were dramatically increased in SAH +vehicle group compared to the sham group ($P < 0.01$, Figure 3). However, DHLA (30 mg/kg) treatment enhanced the level of LAMP1 and inhibited the expression of p-CaMKIIα, NLRP3, cleaved caspase-1 and IL-1β in SAH+DHLA group, when compared to SAH +vehicle group, but not in the sham+DHLA group ($P < 0.05$, Figure 3 and Supplemental Figure III).

DHLA inhibited lysosomal rupture in microglia

The results from double immunofluorescence staining of LAMP1, p-CaMKIIα and Iba-1 indicated that LAMP1 expression in microglia was decreased (Figure 4A), but p-CaMKIIα expression was increased (Figure 4B) after SAH. These alterations were reversed by DHLA treatment in SAH+DHLA group (Figure 4).

Knockdown LAMP1 abolished the anti-inflammation effect of DHLA after SAH

LAMP1 knockdown markedly reversed the neurological improvements of SAH+DHLA (30 mg/kg) rats on modified Gracia and beam balance test at 24 h post SAH. Additionally, there was a significant difference compared with SAH+DHLA+Scr siRNA group ($P < 0.05$, Figure 5A and 5B). Moreover, LAMP1 siRNA intervention significantly inhibited the expression of LAMP1, while increasing p-TAK1, p-JNK, p-CaMKIIα and NLRP3 expression, when compared with SAH+DHLA+Scr siRNA group ($P < 0.05$, Figure 5C).

CaMKIIα siRNA decreased the protein level of p- CaMKIIα, p-TAK1, p-JNK and NLRP3

To study the relationship between CaMKII and TAK1/JNK in regulation of NLRP3 after SAH, CaMKIIα siRNA was administered i.c.v at 48 h before SAH induction. The Western blot results showed that CaMKIIα knockdown markedly increased the neurological score on modified Garcia and beam balance test in SAH+CaMKIIα siRNA group compared with SAH+Scr siRNA group ($P < 0.01$, Figure 6A and 6B). The expression of p-TAK1, p-JNK and NLRP3 were significantly decreased in SAH+CaMKIIα siRNA group compared with SAH+Scr siRNA group ($P < 0.05$, Figure 6C).

Discussion

In the present study, we first found that the expression of LAMP1 decreased, the expression of p-CaMKIIα increased in the brain 24 h after SAH and that they were mainly expressed in neurons and microglia. In addition, the DHLA treatment improved both short and long-term neurofunction after SAH, which were accompanied by an increase in LAMP1 expression and a decrease in p-CaMKIIα and NLRP3 inflammasome expression. Furthermore, knockdown of LAMP1 abolished the neuroprotective effects of DHLA, which were associated with the increased expression of p-CaMKIIα, p-TAK1, p-JNK and NLRP3 inflammasome at 24 h after SAH. Moreover, the knockdown of CaMKIIα downregulated the expression of p-TAK1, p-JNK and NLRP3 and improved neurobehavior at 24 h after SAH. These findings suggested that the administration of DHLA could inhibit lysosomal rupture, attenuate NLRP3 inflammasome activation, and improve neurofunction after SAH at least in part through the LAMP1/CaMKII/TAK1 signaling pathway.

The role of NLRP3 inflammasome in the pathophysiology of EBI after SAH has been well established.[20] Once activated, the NLRP3 inflammasome causes transformation of pro-caspase-1 into cleaved caspase-1 and maturation of IL-1β and IL-18, subsequently contributes to inflammation following SAH.[4] There are three key mechanisms regulating the activation of the NLRP3 inflammasome: the generation of reactive oxygen species (ROS), the efflux of potassium and the rupture of the lysosome.[21] A recent study found that lysosomal rupture triggered cathepsin-dependent protein degradation and activated the

NLRP3 inflammasome.[22] Another study demonstrated that the CaMKII/TAK1/JNK pathway was activated through lysosomal rupture and caused the activation of the NLRP3 inflammasome.[8] However, the mechanisms between lysosomal rupture and NLRP3 activation after SAH have not been elucidated. In the present study, we found that lysosomal rupture can activate the NLRP3 inflammasome through the LAMP1/CaMKII/TAK1 signaling pathway.

LAMP1 is a major component of the lysosomal membrane. LAMP1 was originally thought to protect the lysosomal membrane and was regarded as a lysosomal marker to evaluate the stability of the lysosome state.[9] However, increasing evidence has suggested that LAMP1 has functions beyond the initially suggested roles in maintaining the integrity of the lysosomal membrane.[10] It has been shown that the appearance of LAMP1 accompanied apoptosis. LAMP1 has a crucial role in contributing to the formation of autophagosomes and leads to the progress of autophagy.[11] In our study, we found that the LAMP1 expression decreased and reached the lowest level at 24h after SAH and was mainly expressed on neurons and microglia, indicating an increase of lysosomal rupture after SAH induction. It also revealed some relation between lysosomal rupture and inflammation after SAH, since the activation of microglia is associated with neuroinflammation.[23]

Lipoic acid (LA) and its reduced form, DHLA, have been regarded as effective antioxidant molecules. LA is reduced by lipoamide dehydrogenase to the corresponding DHLA, which is more effective in performing antioxidant functions.[24] The antioxidant properties of LA and DHLA has been shown in many diseases associated with redox status imbalance such as diabetes and cardiovascular diseases.[25] Moreover the neuroprotective effects of LA and DHLA have been shown in many models of central nervous system disease such as ischemic stroke, traumatic brain injury and Alzheimer disease.[26–28] And proper dose of LA has been proved to alleviate oxidative stress and reduce formation of ROS in SAH model.[13] In our study, we found the DHLA treatment significantly improved the short and long-term neurobehavior after SAH. In addition, the middle dosage of DHLA (30mg/kg) significantly increased the expression of LAMP1 and decreased the expression of the NLRP3 inflammasome after SAH, indicating that DHLA inhibited lysosomal rupture and the activation of the NLRP3 inflammasome. It is consistent with a recent reporting that DHLA can stabilize the lysosomal membrane and improve neurofunction.[6] To study whether the anti-inflammation and neuroprotective effects of DHLA are associated with LAMP1 expression, we used LAMP1 siRNA i.c.v injection 48 h before SAH induction. The results showed that knockdown of LAMP1 abolished the neuroprotective effects of DHLA and reversed the expression of downstream proteins, indicating an essential role of lysosomal rupture and LAMP1 in the downstream pathway.

A study *in vitro* showed that lysosomal rupture regulated NLRP3 inflammasome activation through the CaMKII/TAK1/JNK pathway.[8] CaMKII is a sensitive Ca^{2+} receptor. The Ca^{2+} released from lysosomal rupture is sufficient to evoke CaMKII and activate the downstream factors. CaMKIIα is one of the major forms of CaMKII. and it can sustain the activation of CaMKII. CaMKIIα has also been proved to contribute to inflammation in microglia.[29] So in our study we mainly focus on the CaMKIIα and we found the expression of p-CaMKIIα increased after SAH and peaked at 24 h after SAH induction, which is consistent with the

degree of lysosomal rupture. In addition, DHLA, the lysosomal membrane stabilizer, significantly inhibited the expression of p-CaMKIIα. Meanwhile, the LAMP1 siRNA reversed the decreasing level of p-CaMKIIα by DHLA. These results indicate that CaMKII maybe a critical downstream effector of lysosomal rupture and LAMP1. And DHLA treatment may function through LAMP1/CaMKII pathway. However, the western blot and immunohistochemistry staining results of CaMKIIα may have cross-reaction with CaMKIIβ, which has similar structural domain and close relation with CaMKIIα. Since the alpha subunit is the predominant form in forebrain and the beta subunit is the dominant form in the cerebellum,[30] we used whole left hemisphere of the brain in the experiments and believe that CaMKIIα should be responsible for the major function, although there may be partly mixed with some CaMKIIβ. Moreover, the silencing of CaMKIIα significantly improved neurofunction. This result is consistent with a recent study showing that CaMKII inhibitor prevented impaired sensorimotor function after SAH.[31] Additionally, several studies have demonstrated that CaMKII regulates the activity of TAK1/JNK, which is a central molecule in multiple signaling pathways.[32–34] However, little is known about the exact downstream signaling cascade initiated by CaMKII/TAK1/JNK after SAH. In the current study, results showed that following administration of DHLA the level of p-TAK1 and p-JNK markedly decreased, which concurrently decreased p-CaMKIIα expression. In addition, with the CaMKIIα siRNA i.c.v injection, the level of p-TAK1, p-JNK and NLRP3 dramatically decreased. These results suggest that lysosomal rupture and the activation of LAMP1/CaMKII/TAK1 signaling pathway underlies the anti-inflammatory effect of DHLA after SAH.

There are some limitations in the present study. The pathophysiology of lysosomal rupture is complicated. Other pathways may exist in the activation of the NLRP3 inflammasome. Meanwhile, the mechanisms of DHLA-induced-stability of the lysosomal membrane and inhibition lysosomal rupture remain to be studied further.

In conclusion, our study showed that DHLA treatment can improve neurofunction and alleviate inflammation through the LAMP1/CaMKII/TAK1 pathway in early brain injury after SAH. It may provide an optical method in the treatment of EBI after SAH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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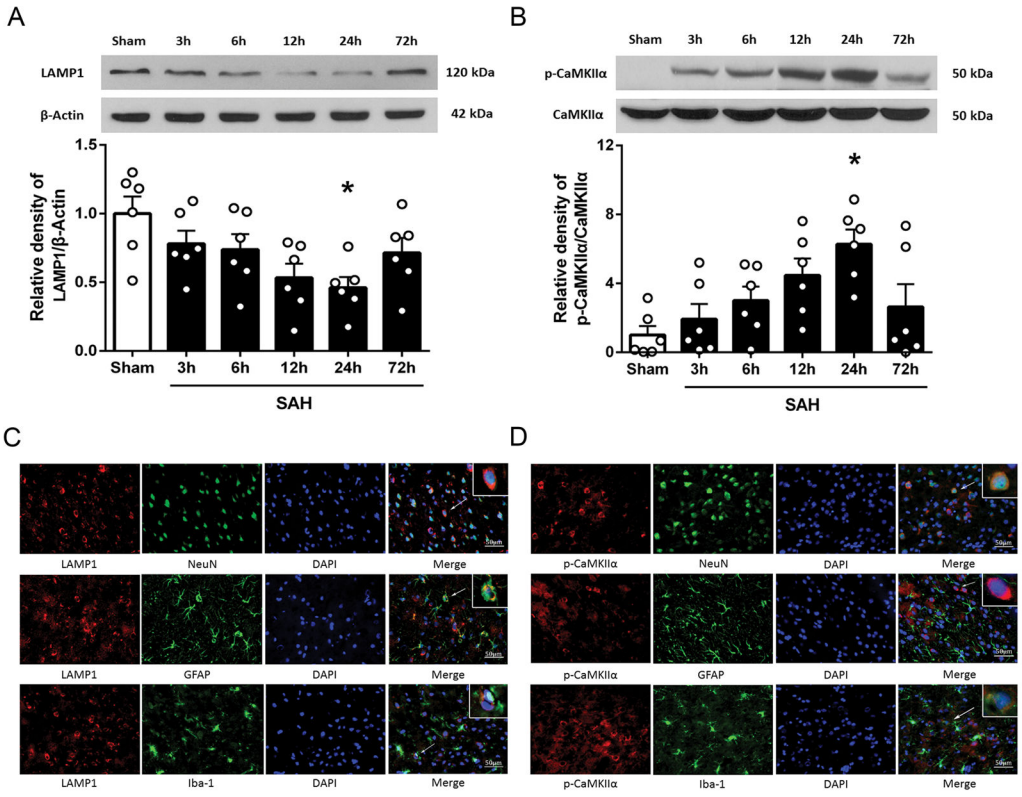

Figure 1. Expression of lysosome-associated membrane protein-1 (LAMP1) and calcium/calmodulin-dependent protein kinase II (p-CaMKIIα) after SAH

(A) Representative Western blot images and quantitative analyses of LAMP1 time course from the left hemisphere after SAH. (B) Representative Western blot images and quantitative analyses of p-CaMKIIα time course from the left hemisphere after SAH. n=6 per group. (C) Double immunofluorescence staining for LAMP1 (red) in the neuron (NeuN, green), astrocytes (GFAP, green) and microglia (Iba-1, green) in the left basal cortex at 24 h after SAH. (D) Double immunofluorescence staining for p-CaMKIIα (red) in the neuron (NeuN, green), astrocytes (GFAP, green) and microglia (Iba-1, green) in the left basal cortex at 24 h after SAH. n=2 per group. *$P < 0.05$ vs. sham group. Bars represent mean ± SEM. Scale bar = 50 μm.

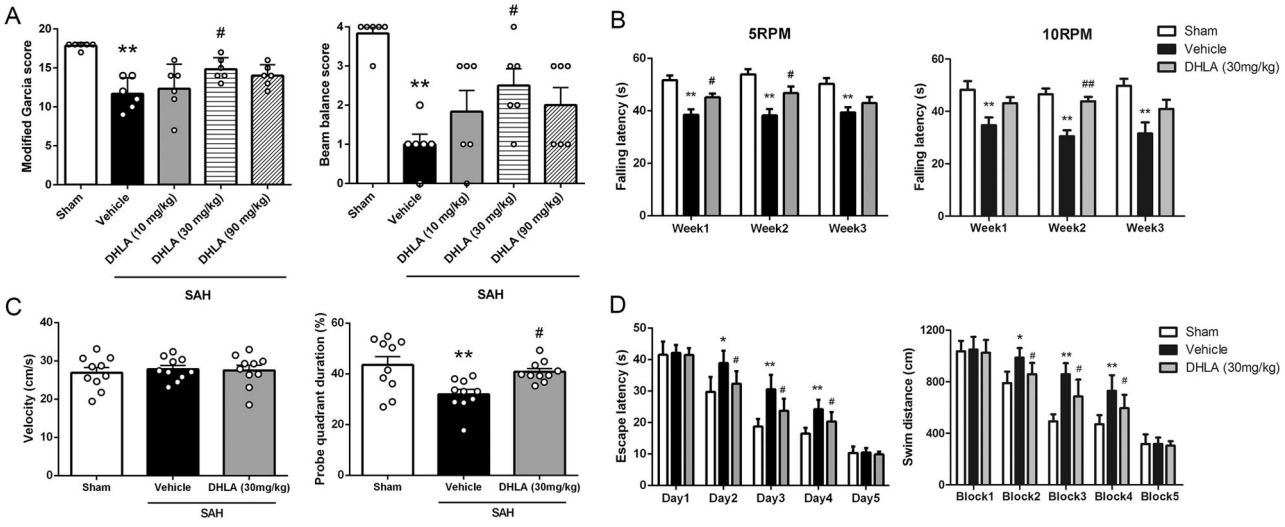

Figure 2. Dihydrolipoic acid (DHLA) improved the short and long-term neurobehavior after SAH

(A) Modified Garcia score and beam balance score. n=6 per group. (B) Rotarod test of 5RPM and 10RPM. (C) Velocity and probe quadrant duration of water maze test. (D) Escape latency and swim distance of water maze test. n=10 per group. ***P** < 0.05, ****P** < 0.01 vs. Sham group; #*P* < 0.05, ##*P* < 0.01 vs. SAH+vehicle group. Bars represent mean ± SEM.

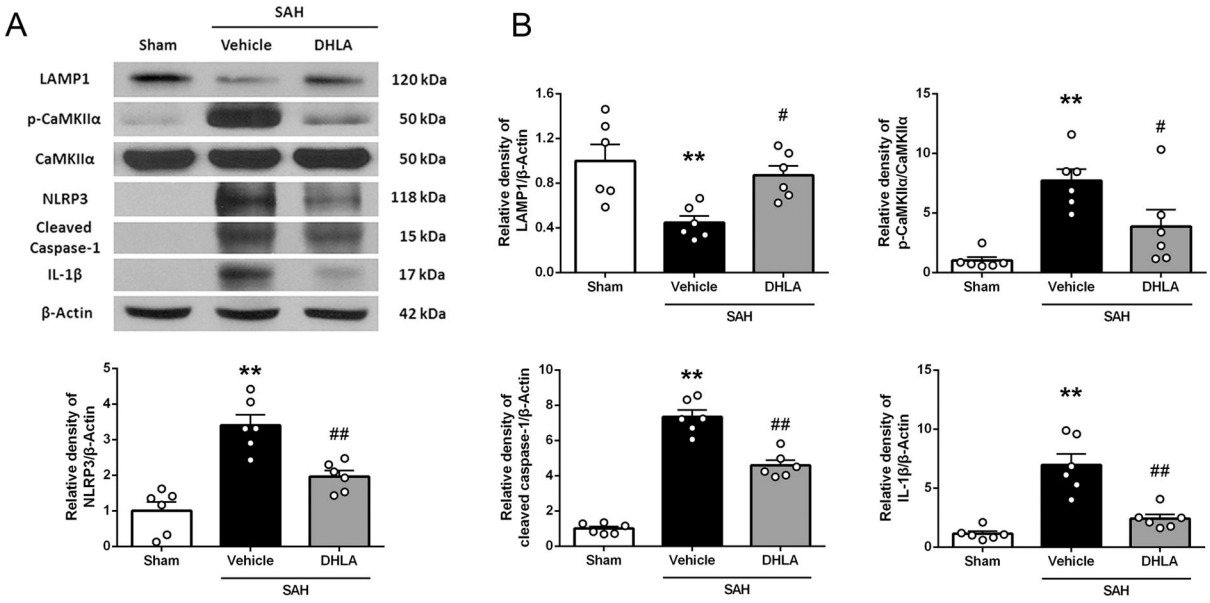

Figure 3. The effects of DHLA in the protein expression of LAMP1, p-CaMKIIα, NLRP3, Cleaved caspase-1, IL-1β

(A) Representative Western blot images. (B) Quantitative analyses of LAMP1, p-CaMKIIα, NLRP3, Cleaved caspase-1, IL-1β. n= 6 per group. **$P < 0.01$ vs. Sham group; #$P < 0.05$, ##$P < 0.01$ vs. SAH+vehicle group. Bars represent mean ± SEM.

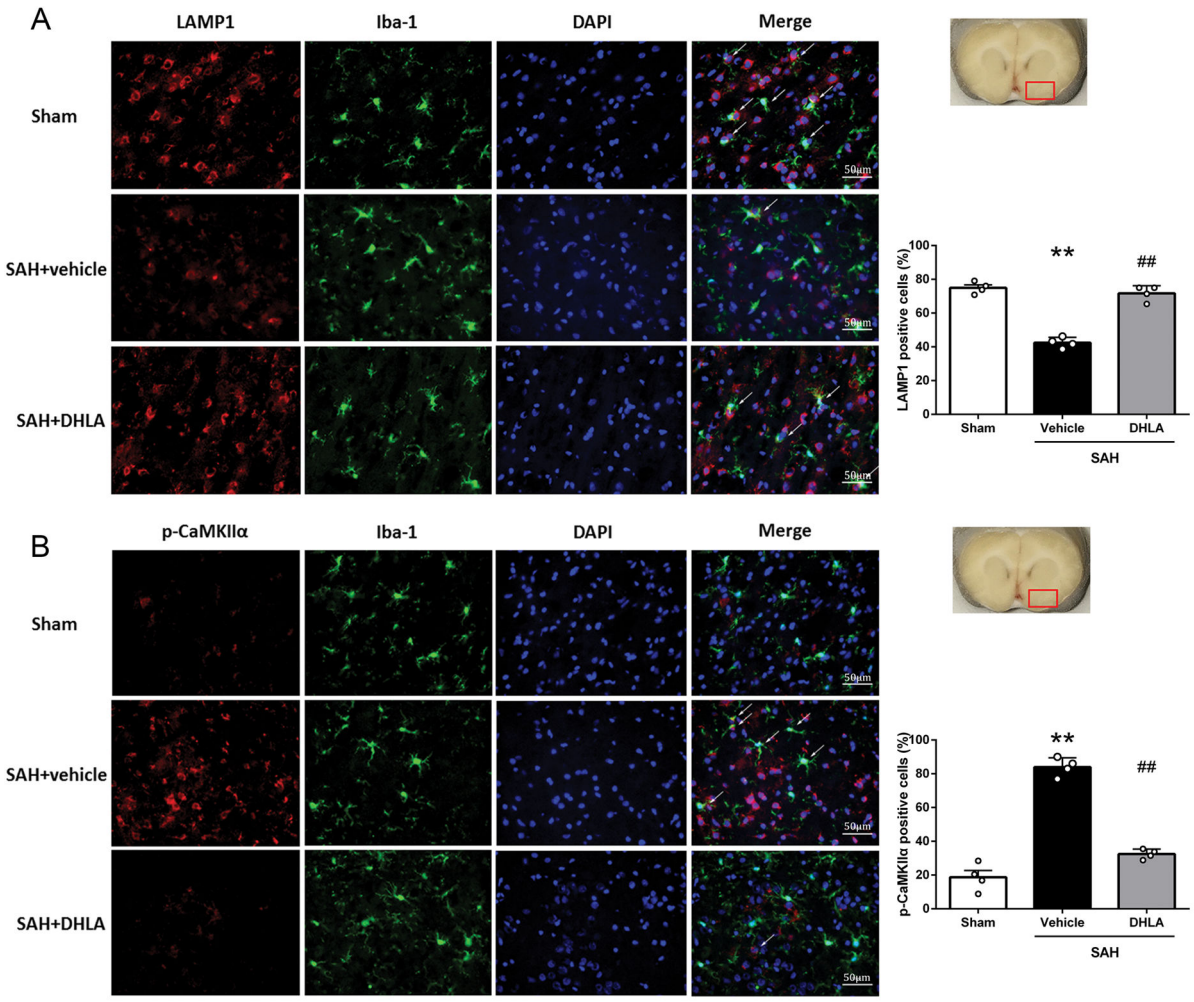

Figure 4. The effects of DHLA in the double immunofluorescence staining of LAMP1 and p-CaMKIIα

(A) Double immunofluorescence staining for LAMP1 (red) in microglia (Iba-1, green) and quantitative analyses of LAMP1. (B) Double immunofluorescence staining for p-CaMKIIα (red) in microglia (Iba-1, green) and quantitative analyses of p-CaMKIIα. n=4 per group. **$P < 0.01$ vs. Sham group; ##$P < 0.01$ vs. SAH+vehicle group. Bars represent mean ± SEM. Scale bar = 50 μm.

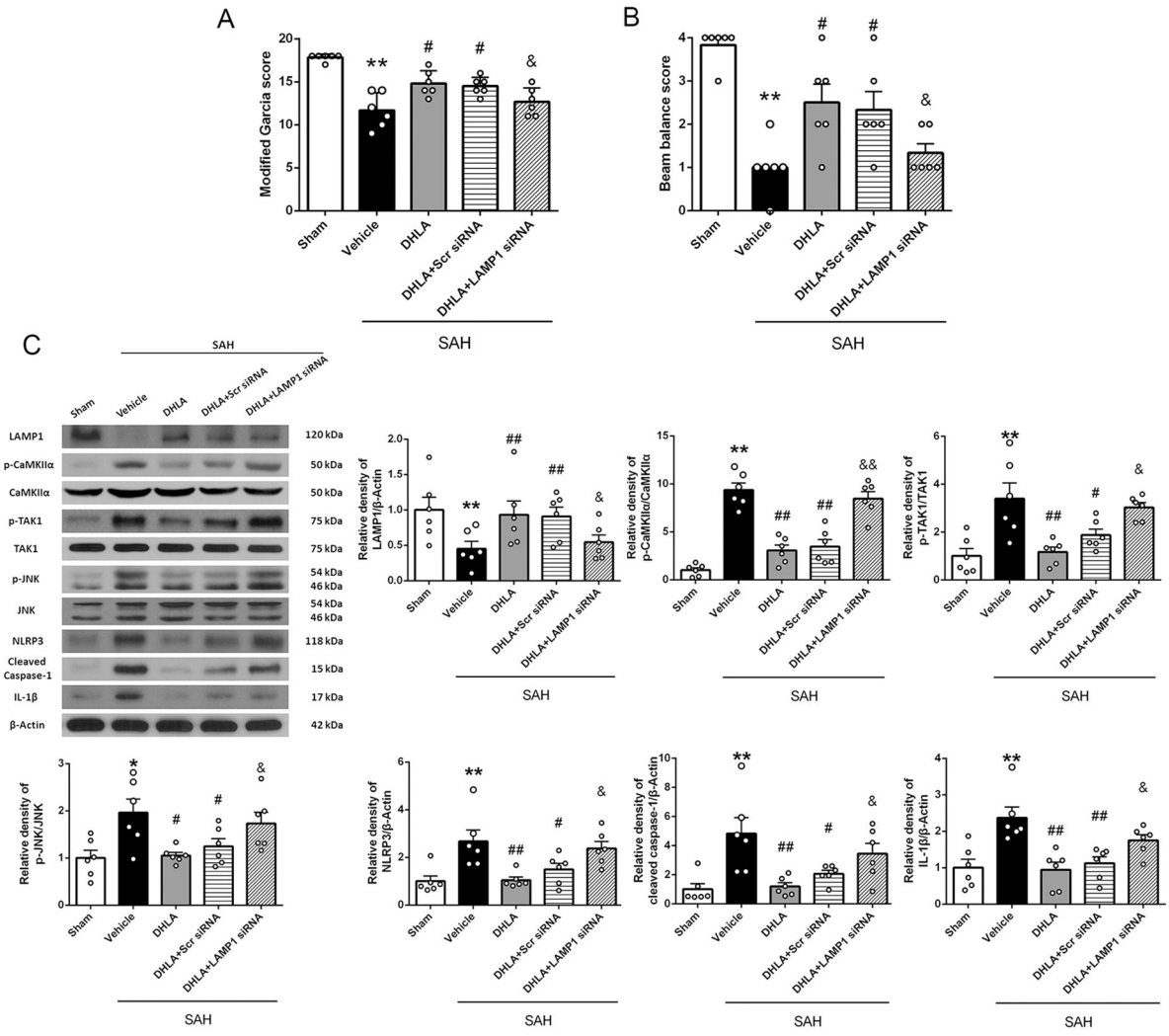

Figure 5. Knockdown LAMP1 abolished the anti-inflammation effect of DHLA after SAH

(A) Modified Garcia score. (B) Beam balance score. (C) Representative Western blot images and quantitative analyses of LAMP1, p-CaMKIIα, p-TAK1, p-JNK, NLRP3, cleaved caspase-1and IL-1β. n=6 per group. **$P < 0.05$, **$P < 0.01$ vs. Sham group; #$P < 0.05$, ##$P < 0.01$ vs. SAH+vehicle group. & $P < 0.05$, && $P < 0.01$ vs. SAH+DHLA+Scr siRNA group. Scr, scramble. siRNA, small interfering ribonucleic acid Bars represent mean ± SEM.

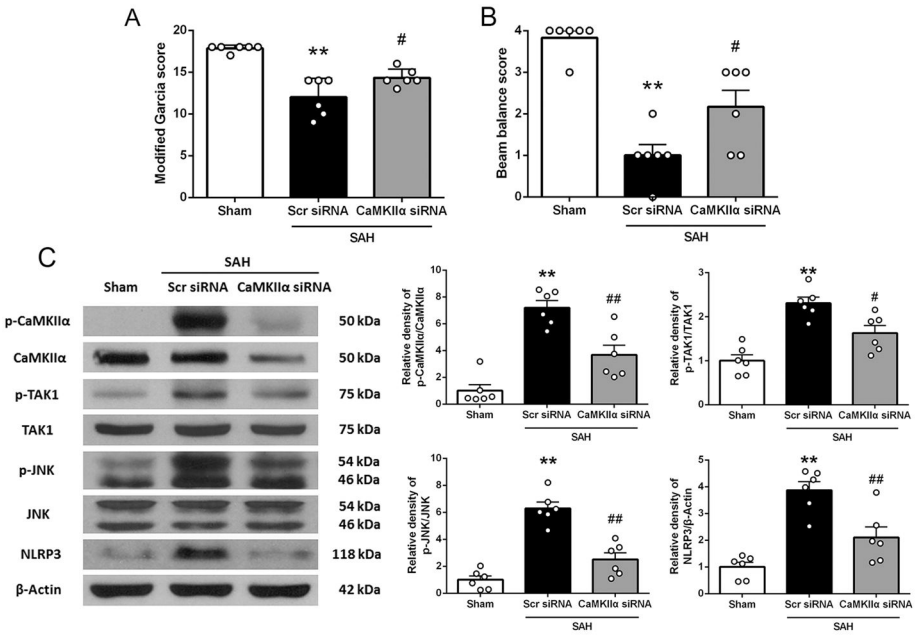

Figure 6. The effects of CaMKIIα siRNA on the neurological function and the protein expression of p- CaMKIIα, p-TAK1, p-JNK and NLRP3

(A) Modified Garcia score. (B) Beam balance score. (C) Representative Western blot bands and quantitative analyses of p-CaMKIIα, p-TAK1, p-JNK, NLRP3. n=6 per group. ***P* < 0.01 vs. Sham group; #*P* < 0.05, ##*P* < 0.01 vs. SAH+Scr siRNA group. Bars represent mean ± SEM.