Tamibarotene Improves Hippocampus Injury Induced by Focal Cerebral Ischemia-Reperfusion via Modulating PI3K/Akt Pathway in Rats

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Goal: The present study aimed to examine whether Am80 (tamibarotene) protects the hippocampus against cerebral ischemia-reperfusion (I/R) injury and whether phosphoinositide-3-kinase/Akt (PI3K/Akt) pathway mediates this effect. *Materials and methods:* Rats were subjected to 90 minutes of middle cerebral artery occlusion followed by 24 hours of reperfusion. The animals were randomly divided into 7 groups: sham-operated group; I/R group; groups pretreated with 2 mg/kg, 6 mg/kg, and 10 mg/kg of Am80; Am80 (6 mg/kg) combined with the selective PI3K inhibitor wortmannin (0.6 mg/kg), and wortmannin (0.6 mg/kg) only group. After 24 hours of reperfusion, neurological deficits and infarct volume were measured. Pathological changes in hippocampal neurons were analyzed by transmission electron microscopy. Neuronal survival was examined by TUNEL staining. The expression of Bcl-2, Bax, and Akt, and Akt phosphorylation (p-Akt) were measured by Western blotting and quantitative real-time polymerase chain reaction. *Findings:* The pretreatment with Am80 improved the neurologic deficit score, reduced infarct volume, and decreased the number of TUNEL-positive cells in the hippocampus. Moreover, Am80 pretreatment downregulated the expression of Bax, upregulated the expression of Bcl-2, and increased the level of p-Akt. Wortmannin abolished in part the increase in p-Act and the neuroprotective effect exerted on the ischemic by Am80 pretreatment. *Conclusions:* Our results documented that Am80 pretreatment protects ischemic hippocampus after cerebral I/R by regulating the expression of apoptosis-related proteins through the activation of the PI3K/Akt signaling pathway.

Key Words: Tamibarotene—Am80—hippocampus injury—cerebral ischemia-reperfusion—apoptosis—PI3K/Akt pathway

Introduction

Ischemic stroke is the second cause of death and the most frequent reason for long-term disability.[1] Restoration of blood flow by thrombectomy or thrombolytic agents during the optimal time window reduces functional deficits in stroke patients. However,

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reperfusion results in the formation of reactive oxygen species and free radicals, leading to reperfusion injury.[2] In spite of extensive studies focused on the prevention of reperfusion injury, few neuroprotective agents identified in basic research have been successfully applied clinically. Thus, it becomes critical to elucidate the exact molecular mechanisms underlying the pathogenesis of cerebral ischemia/reperfusion (I/R) injury in order to develop new effective therapies.

Apoptosis resulting from ischemic stroke is considered to represent the critical mode of neuronal cell death.[3,4] The mechanism of most types of apoptosis involves the mitochondria. With the activation of apoptotic signaling and generation of an oxidative load in mitochondria, the permeability of their outer membrane increases, leading to the translocation of proapoptotic protein Bax from the cytosol to the mitochondria This translocation is controlled by the Bcl-2 family of proteins[5,6] and results in the release of cytochrome c into the cytosol.[7] The presence of cytochrome c in the cytoplasm triggers the formation of the apoptosome complex, which induces DNA fragmentation and apoptotic neuronal cell death.[8] Thus, the development of preventive or therapeutic measures targeting this mechanism of apoptosis after I/R is of crucial importance.

Am80, a selective agonist of retinoic acid receptor α (RARα) has been approved in Japan for the treatment of acute promyelocytic leukemia.[9] Am80 has also been considered to be a promising candidate drug for the prevention of Alzheimer's disease since it controls the transcription of multiple genes involved in etiology and pathology of this condition.[10] The anti-inflammatory effect of Am80 was demonstrated to ameliorate the symptoms of experimental autoimmune encephalomyelitis.[11,12] It also improved the recovery in rats with spinal cord injury[13] and ameliorated myocardial I/R damage by reducing apoptosis of cardiomyocytes.[14] Finally, and most importantly, Am80 was documented to rescue neurons, attenuate inflammatory reactions, and improve behavioral recovery after intracerebral hemorrhage in mice.[15]

Despite these promising findings, whether Am80 can have a beneficial impact on I/R-induced hippocampus injury remains unknown. Here, we investigated the effects of Am80 on hippocampus injury induced by cerebral ischemia in rats using the middle cerebral artery occlusion (MCAO) model. The underlying mechanisms were analyzed as well.

Materials and Methods

Experimental Animals

Adult male Sprague-Dawley (SD) rats, weighing 260-280 g, were obtained from the Department of Experimental Animals, Chongqing Medical University (Chongqing, China). The animals were housed under specific pathogen-free conditions. All procedures involving the rats were approved by the Experimental Ethics Committee of Chongqing Medical University (Permit No. SCXK (Chongqing) 2007-0001) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The surgical procedures were performed under anesthesia, and all efforts were made to minimize the suffering of the animals.

Drug Treatment and the MCAO Model

One hundred eighty rats were used in the present study. Transient focal cerebral ischemia was induced by MCAO as previously described.[16] Briefly, after the rats were anesthetized with 4% pentobarbital sodium, the right common carotid artery, internal carotid artery, and external carotid artery were carefully separated under an operating microscope. To induce the I/R event, a suture was inserted into the internal carotid artery through the external carotid artery stump, gently pushed forward to occlude the middle cerebral artery, and withdrawn 90 minutes later to restore perfusion. The sham-operated rats were subjected to an identical procedure except that the suture was not inserted. The overall mortality rate associated with surgery was 3.33%. During the entire procedure, rectal temperature was maintained at 37 ± 0.5°C using a thermistor-controlled heating pad and head heating lamp. After 24 hours of reperfusion, deficits in neurological function were scored, and only the animals that scored from 1 to 4 were chosen for further experiments. The rats that had brain hemorrhage were excluded from the study, regardless of the presence or absence of ischemia.

The rats were randomly divided into 7 groups (n = 10, in each group): (1) sham-operated group, (2) I/R group, (3) I/R + Am80 (2 mg/kg) group, (4) I/R + Am80 (6 mg/kg) group, (5) I/R + Am80 (10 mg/kg) group. (6) Am80 (6 mg/kg) + wortmannin (0.6 mg/kg) group, (7) wortmannin (0.6 mg/kg) group. Am80 (Ark Pharm. Inc., IL) was dissolved in 0.5% carboxymethyl cellulose solution.[11,12] Am80 at the corresponding concentration was administered by gavage once a day for 7 days, with the last dose given 2 hours before the surgery. The sham-operated group and the I/R group received an equal volume of 0.5% carboxymethyl cellulose solution. Animals in the groups receiving wortmannin were injected intravenously with 0.6 mg/kg of the drug at 30 minutes before the surgery.[17]

Evaluation of Ischemic Outcomes

At 24 hours after the onset of ischemia, neurological deficit scores were established by a blinded investigator using the previously described modified Longa's method.[18] The deficit was scored according to the following 5-point scale:

Score *0* = no neurological deficit symptoms, both forelimbs display normal strength and can be extended straight.
Score *1* = failure to fully extend the left forepaw.

Score *2* = circling to the left, but preservation of normal posture at rest.
Score *3* = leaning to the left when walking.
Score *4* = absence of spontaneous walking and a low level of consciousness.

To quantify the volume of cerebral infarct, rats were sacrificed with aether, the brains were quickly removed, frozen, and cut into 5 coronal sections, 2-mm thick. The sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride solution at 37°C for 15 minutes and fixed overnight in 4% paraformaldehyde. The infarct volume was quantitated using ImagePro Plus software and expressed as the percentage of contralateral hemisphere.

TUNEL Staining

Apoptosis of neuronal cells was determined in paraffin-embedded sections by TUNEL staining using the in situ cell death detection kit (Roche, Mannheim, Germany). The procedure was performed according to the manufacturer's instructions. The nuclei of apoptotic cells were stained brown. The number of apoptotic cells with brown-stained nuclei and the total number of cells was counted in 5 different regions. Apoptotic index was calculated as previously described.[19]

Transmission Electron Microscopy (TEM)

The structure of the ischemic tissue was analyzed by TEM. For this purpose, rats were deeply anesthetized and perfusion-fixed by with 4% paraformaldehyde and 1% glutaraldehyde. The CA1 region of the hippocampus was cut into 1 cubic millimeter fragments and fixed in 2.5% glutaraldehyde and 1% osmic acid for 4 hours. Specimens were dehydrated with acetone and embedded in Epon812. Ultrathin sections of the specimens were cut and stained with uranyl acetate and lead citrate. The samples were observed under the Hitachi 7100 TEM (Hitachi, Japan).

Western Blot Analysis

Total proteins were extracted from hippocampal tissue by lysis buffer (Beyotime Biotechnology, China), and protein concentration was measured with the bicinchoninic acid protein assay kit (P0012S, Beyotime, China). Proteins were separated by 10% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (P0012A, Beyotime, China) and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% bovine serum albumin for 1 hours at room temperature and incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal antibodies against RARα (2554S, Cell Signaling Technology, Danvers, Massachusetts, diluted 1:1000), Akt (9272, Cell Signaling Technology, diluted 1:1000), Bcl-2 (ab59348, Abcam, Cambridge, United Kingdom, diluted 1:1000), mouse monoclonal antibody against Bax (60267-1-Ig, Proteintech, Wuhan, China, diluted 1:1000), and rabbit monoclonal antibodies against phospho-Ser-473-Akt (p-Akt) (4060, Cell Signaling Technology, diluted 1:1000) and β-actin (4970, Cell Signaling Technology, diluted 1:2000). Subsequently, the membranes were washed 3 times and incubated with horseradish peroxidase, a secondary goat anti-rabbit or anti-mouse antibodies, diluted 1:3000, for 1 hour at room temperature. The immune reactivity was revealed by enhanced chemiluminescence, the membranes were scanned with the gel imaging apparatus (Bio-Rad, Hercules, CA), and the intensity of the bands was analyzed using Image Lab (Bio-Rad).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was used to measure the expression of Bcl-2 and Bax at the mRNA level. Total RNA of the hippocampus was extracted using Trizol RNA extraction kit (TaKaRa Bio Inc., Japan), and cDNA was prepared by reverse transcription using the PrimeScript TM RT Reagent Kit with gDNA Eraser (TaKaRa Bio Inc.) according to the manufacturer's protocol. The target genes and the corresponding PCR primers are listed in Table 1. Amplification, detection, and quantification were carried out with the CFX96 Real-Time PCR Detection System (Bio-Rad) in the presence of the fluorescent dye SYBR Green (TaKaRa Bio Inc.). The thermocycling conditions included 95°C for 30 seconds followed by 39 cycles of denaturation at 95°C for 5 seconds and annealing and extension at 60°C for 30 seconds. Each sample was analyzed at least 3 times. All threshold cycle values of target genes were normalized to β-actin and relative expression was calculated using the $2^{-\Delta\Delta_{ct}}$ method.

Statistical Analysis

The data are expressed as the mean ± SEM. All statistical analyses were performed using the SPSS 17.0 software. Comparisons among multiple groups were calculated by one-way analysis of variance (ANOVA) followed by Tukey's test if ANOVA indicated the presence of a significant difference. Two-way ANOVA was used to analyze the effects of multiple factors. The neurological deficit scores were analyzed using the Mann-Whitney *U* test or Kruskal-Wallis test, followed by Steel-Dwass test for multiple comparisons. *P* values less than .05 were considered statistically significant.

Table 1. *Sequences of primers used in qRT-PCR*

Gene	*Primer*	*Sequence*
Bcl-2	forward	5′-GACTGAGTACCTGAACCGG-CATC-3′
	reverse	5′-CTGAGCAGCGTCTTCAGA-GACA-3′
Bax	forward	5′-AGACACCTGAGCT-GACCTTGGA-3′
	reverse	5′-TTGAAGTTGCCATCAG-CAAACA-3′
β-actin	forward	5′-ACGGTCAGGTCATCAC-TATCG-3′
	reverse	5′-GGCATAGAGGTCTTTACG-GATG-3′

Results

Am80 Alleviates I/R-Induced Brain Injury in Rats

To determine whether Am80 protects the brain against I/R injury, the neurological function was assessed at 24 hours after reperfusion. The rats in I/R group displayed severe neurological deficits (Fig 1a). The pretreatment with Am80 at 6 and 10 mg/kg, but not 2 mg/kg, significantly decreased the neurological deficit score, suggesting that Am80 pretreatment improves in a dose-dependent manner the functional outcome after cerebral I/R injury.

The staining of consecutive brain sections with 2,3,5-triphenyltetrazolium chloride revealed the absence of cerebral infarction in the sham-operated animals, as evidenced by deep-red staining. Large infarct areas, documented by pale-green staining, were present in brains subjected to I/R. However, the pretreatment with Am80 at 6 and 10 mg/kg, but not 2 mg/kg, significantly decreased the volume of cerebral infarct (Fig 1b,c). These findings demonstrate that Am80 decreases in a dose-dependent manner the extent of brain injury caused by the I/R.

Am80 Alleviates Neuronal Ultrastructural Injury Induced by I/R

TEM imaging documented neuronal injury in ischemic brain tissue at the ultrastructural level. As shown in Figure 2, neuronal mitochondria and nuclear chromatin displayed normal appearance in the sham-operated group. Conversely, severe karyolysis and cytoplasm cavitation were present in neurons of the hippocampal CA1 region in rats subjected to I/R. However, Am80 pretreatment significantly decreased the I/R-induced ultrastructural damage of neurons.

Am80 Reduces I/R-Induced Apoptosis and Apoptosis-Related Proteins

Fragmentation of nuclear DNA, typical of early apoptosis, was detected by the TUNEL staining. TUNEL-positive cells were essentially absent in brains of the sham-operated rats (Fig 3). In contrast, numerous cells located in the ischemic area of animals subjected to I/R were TUNEL-positive. However, the pretreatment with Am80 significantly reduced the number of TUNEL-positive cells (Fig 3).

To further investigate the effect of Am80 on the activation of apoptosis in the hippocampal region, the expression of 2 apoptosis-associated factors, Bcl-2 and Bax, was studied. This analysis was done at the protein and mRNA levels by, respectively, Western blotting and qRT-PCR. The expression of Bcl-2 protein in ischemic rat brain was downregulated at 24 hours after the I/R injury, but pretreatment with Am80 increased Bcl-2 expression (Fig 4a, b). The expression of Bcl-2 mRNA did not follow the same pattern and was comparable in sham-operated rats and rats in I/R group. The expression of Bcl-2 protein in ischemic rat brain was downregulated at 24 hours after the I/R injury, but pretreatment with Am80 increased Bcl-2 expression (Fig 4a, b). However, the 6 mg/kg and 10 mg/kg doses of Am80 clearly increased the expression of Bcl-2 at the mRNA level in a dose-dependent manner (Fig 4d). In

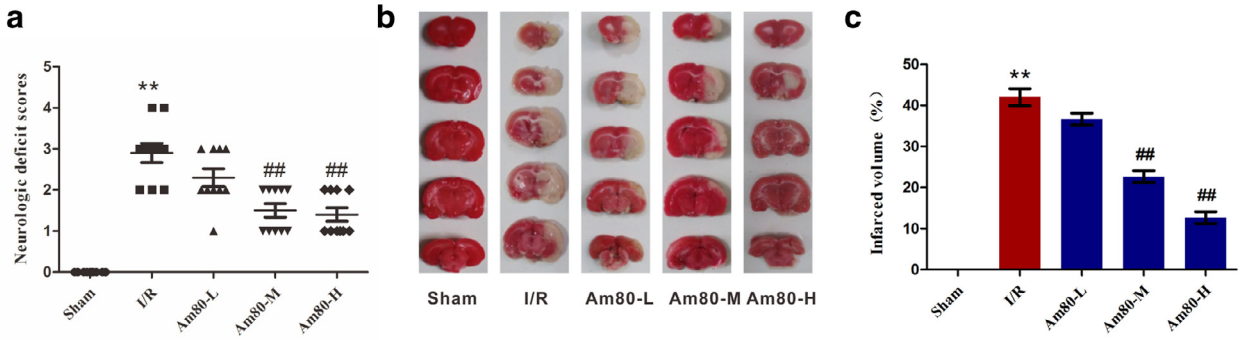

Figure 1. ***Am80 alleviates focal ischemia-induced brain injury in rats.*** *(a) Neurological deficit scores in rats subjected to I/R in the 5 groups (n = 10 per group). (b) Representative images of TTC-stained brain sections from different groups. (c) Infarct volume of rats brains presented as a percentage of an intact hemisphere (n = 3). Values are expressed as the mean ± SEM. ANOVA* $^{**}P < 0P.01$ *versus sham, and* $^{\#\#}P < .01$ *versus I/R. Abbreviation: ANOVA, analysis of variance; I/R, ischemia-reperfusion; TTC, triphenyltetrazolium chloride.*

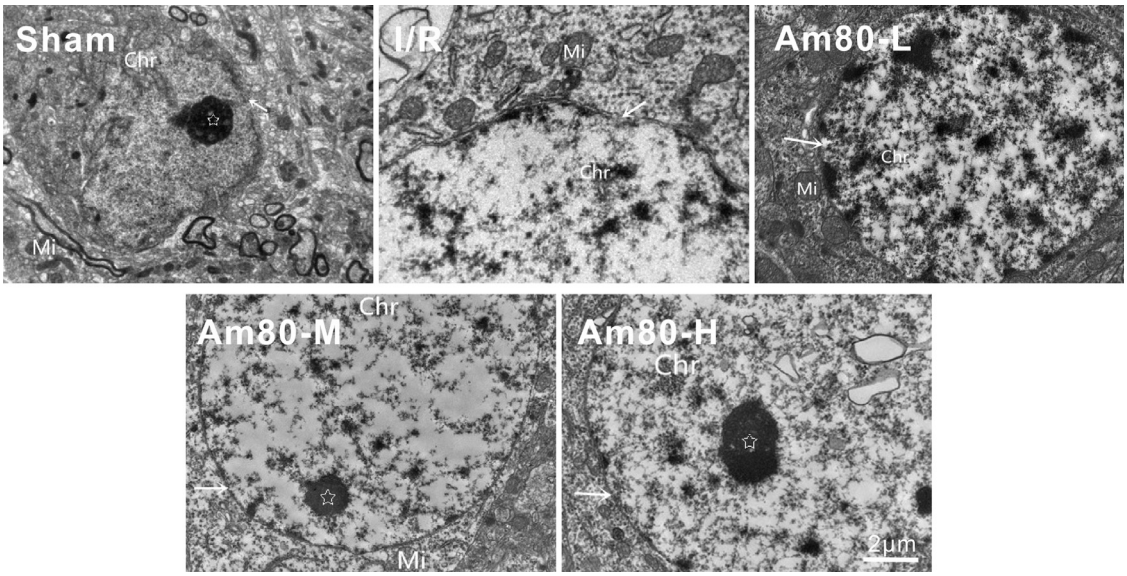

Figure 2. ***Am80 alleviates I/R-induced ultrastructural injury in neurons.*** *Representative TEM images of karyolysis and cytoplasm cavitation in the hippocampal CA1 region in the rat brain exposed to I/R (n = 3). Scale bar = 2 μm. Abbreviation: I/R, ischemia-reperfusion; TEM, transmission electron microscopy.*

contrast to Bcl-2, the expression of Bax at both protein and mRNA level was significantly upregulated in I/R group. The pretreatment of rats with Am80 markedly decreased the Bax protein and mRNA expression, and this downregulation was dose-dependent (Fig 4c, e). Thus, Am80 modulates the expression of Bcl-2 and Bax in a manner that may protect neuronal cells from apoptosis and alleviate I/R-induced brain injury.

Am80 Increases p-Akt Expression After I/R Injury

The expression of Akt and p-Akt in the hippocampus was evaluated by Western blotting to determine whether this protein and its phosphorylation state were involved in cerebral I/R injury. As shown in Figure 5, the expression of total Akt was comparable in all experimental groups. Importantly, the level of Akt phosphorylation was significantly reduced after I/R injury. However,

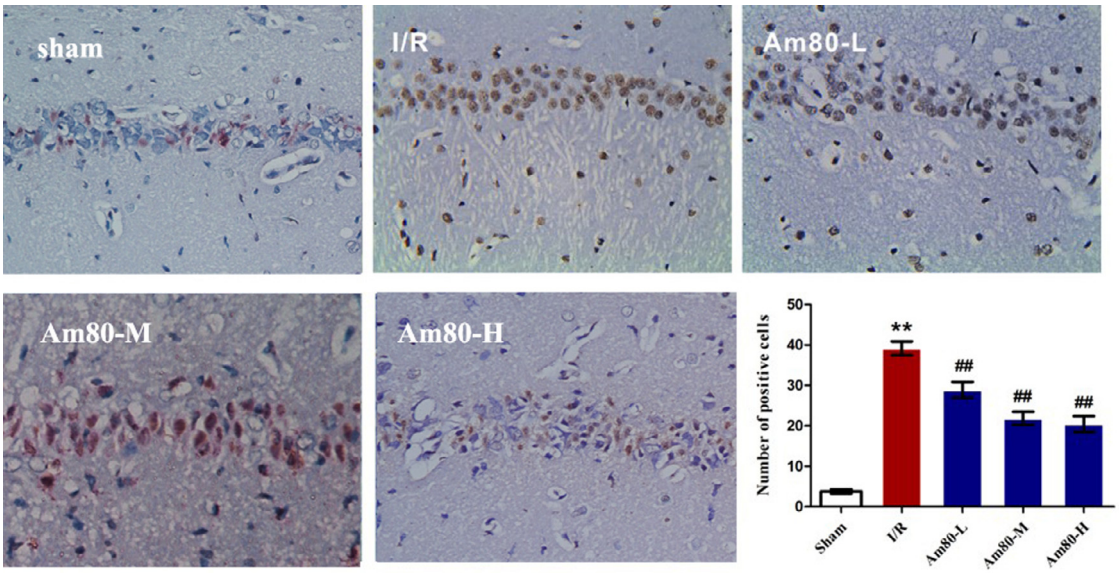

Figure 3. ***Am80 treatment attenuates apoptosis induced by I/R.*** *Representative microphotographs of TUNEL in the cerebral hippocampus 24 hours after MCAO. The number of TUNEL-positive cells in the I/R group is significantly increased in comparison with the sham-operated group. After the AM80 treatment, the number of TUNEL-positive cells was evidently suppressed. n = 3, scale bar = 50 μm. Values are expressed as the mean ± SEM. ANOVA $^{**}P < .01$ versus sham, and $^{\#\#}P < .01$ versus I/R. Abbreviation: ANOVA, analysis of variance; I/R, ischemia-reperfusion; MCAO, middle cerebral artery occlusion.*

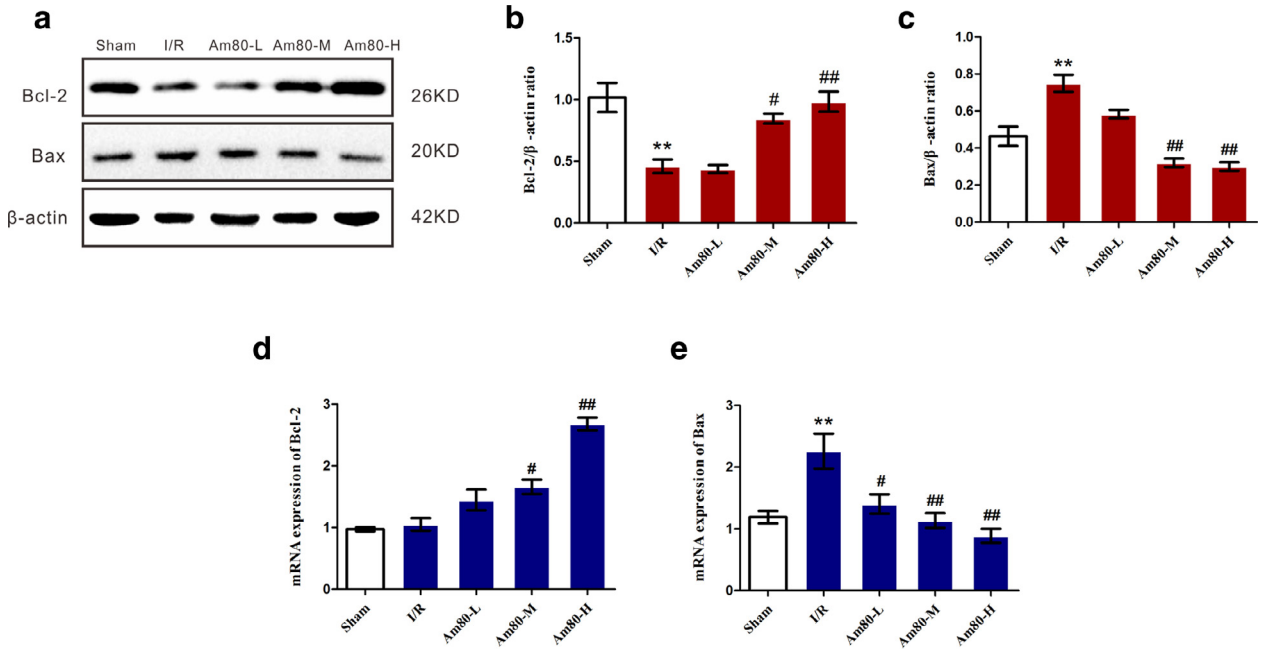

Figure 4. ***Effects of Am80 treatment on the expression of apoptosis-related proteins in Hippocampus.*** *(a) Representative Western blot analysis of Bcl-2 and Bax; (b) quantitative analysis of Bcl-2 protein; (c) quantitative analysis of Bax protein; (d) quantitative analysis of Bcl-2 mRNA (d); (e) quantitative analysis of Bax mRNA. Am80 treatment up-regulated the expression of Bcl-2 and down-regulated the expression of Bax, in particular in the group receiving 10 mg/kg of Am80 (n = 6). Values are expressed as the mean ± SEM. ANOVA $^{**}P < .01$ versus sham and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus I/R. Abbreviation: ANOVA, analysis of variance; I/R, ischemia-reperfusion.*

Am80 pretreatment markedly increased the level of p-Akt in ischemic rat brain.

Am80 Alleviates I/R-Induced Brain Injury in a PI3K-Dependent Manner

To establish whether the PI3K/Akt pathway is involved in I/R injury, rats were treated with wortmannin, an irreversible PI3K inhibitor, at 30 minutes before MCAO, and neurological deficits, infarct size, and levels of Bcl-2, Bax, and p-Akt were measured at 24 hours after reperfusion, respectively. In comparison with the rats treated only with Am80 (6 mg/kg), animals treated with Am80 and wortmannin displayed clear symptoms of severe neurological dysfunction and increased infarct size (Fig 6a, b), suggesting that inhibition of PI3K exacerbated the ischemic brain injury. Additionally, when compared with the groups treated only with Am80, rats treated with the combination of Am80 and wortmannin had decreased level of antiapoptotic protein Bcl-2 and increased level of proapoptotic protein Bax (Fig 6d, e), and inhibited phosphorylation of Akt (Fig 7) at 24 hours after the induction of I/R injury. Of note, rats treated with Am80 and wortmannin had infarct sizes and neurological deficit scores comparable to those seen in I/R group (Fig 6a-c). These data suggested that activation of the PI3K/Akt pathway mediates the neuroprotective effect of Am80 in cerebral I/R injury.

Discussion

The present study tested the hypothesis that Am80 exerts a protective effect on hippocampus following injury induced by cerebral I/R. Previous investigations have demonstrated that ischemic condition in the rat brain results in the formation of an infarct and triggers neurological deficits. Apoptosis is the major type of neuronal cell death after ischemic stroke. The main finding of the current work is that Am80 protects hippocampus injury induced by I/R by modulating the expression of proapoptotic and antiapoptotic proteins through the activation of the PI3K/Akt signaling pathway.

Ischemic stroke involves the obstruction of blood vessels, typically of the middle and anterior cerebral arteries, and reperfusion after ischemia is crucial to restoring the normal function of the brain.[20] However, the restoration of blood flow after a period of ischemia leads to additional severe injury.[21] Since cerebral I/R injury peaks at 24 hours after reperfusion,[22] we elected to test the effect of Am80 on cerebral damage at that time. Over the decades, extensive research has been devoted to advance the progress of therapies for ischemic stroke. Although a large number of agents have been reported to decrease cerebral injury experimentally, but most of them have failed to be clinically applicable due to their side effect.[23,24] Therefore, further studies on the pathophysiology of cerebral ischemia and identification of new targets and effective drugs are urgently needed to benefit public health.

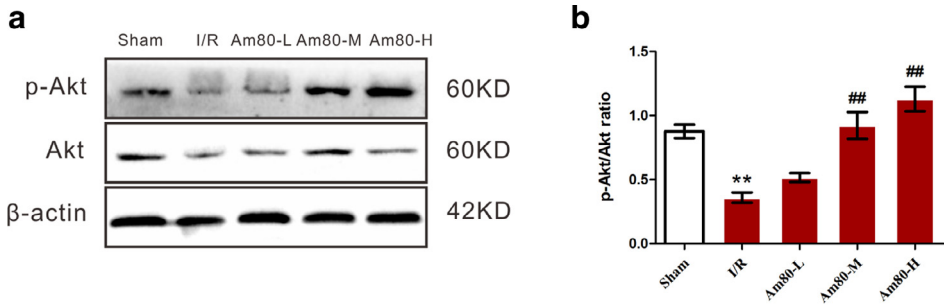

Figure 5. ***Am80 increases the expression of p-Akt 24 hours after the I/R.*** *(a) Representative blot showing the effect of Am80 on phosphorylation of Akt. (b) p-Akt band density relative to the total Akt. Values are expressed as the mean ± SEM. n = 10, ANOVA $^{**}P < .01$ versus sham, and $^{\#\#}P < .01$ versus I/R. Abbreviation: ANOVA, analysis of variance; I/R, ischemia-reperfusion; TTC, triphenyltetrazolium chloride.*

Am80 is a synthetic retinoid which has been approved in Japan for the treatment of acute promyelocytic leukemia. It is an agonist of RAR characterized by a high specificity for RARα and RARβ subtypes, and low binding to the RARγ subtype.[10] Am80 has been shown to exert an anti-inflammatory effect that improved the outcomes of experimental autoimmune encephalomyelitis and intracerebral hemorrhage in mice.[11,15] This drug also exhibits antiapoptotic function in the myocardial I/R injury.[14] Additionally, Am80 has been demonstrated to abrogate motor dysfunction resulting from spinal cord injury in rats; this effect was mediated by improving cell viability and differentiation of neural stem cells.[13] In the present study, administration of Am80 significantly attenuated neurological deficits following 24 hours of reperfusion after MACO. This result is in agreement with previous work demonstrating an improvement in neurologic function afforded by the pretreatment with Am80 in intracerebral hemorrhage models.[15]

Infarct volume is frequently used to measure the extent of neuroprotection following ischemia, and the volume fraction occupied by the infarct reflects the severity of brain damage.[25] The present investigation documented a remarkable reduction in infarct volume by pretreatment with 10 mg/kg of Am80. This result implies that Am80 is a possible candidate drug for the reduction of infarct volume and improvement of neurological outcomes.

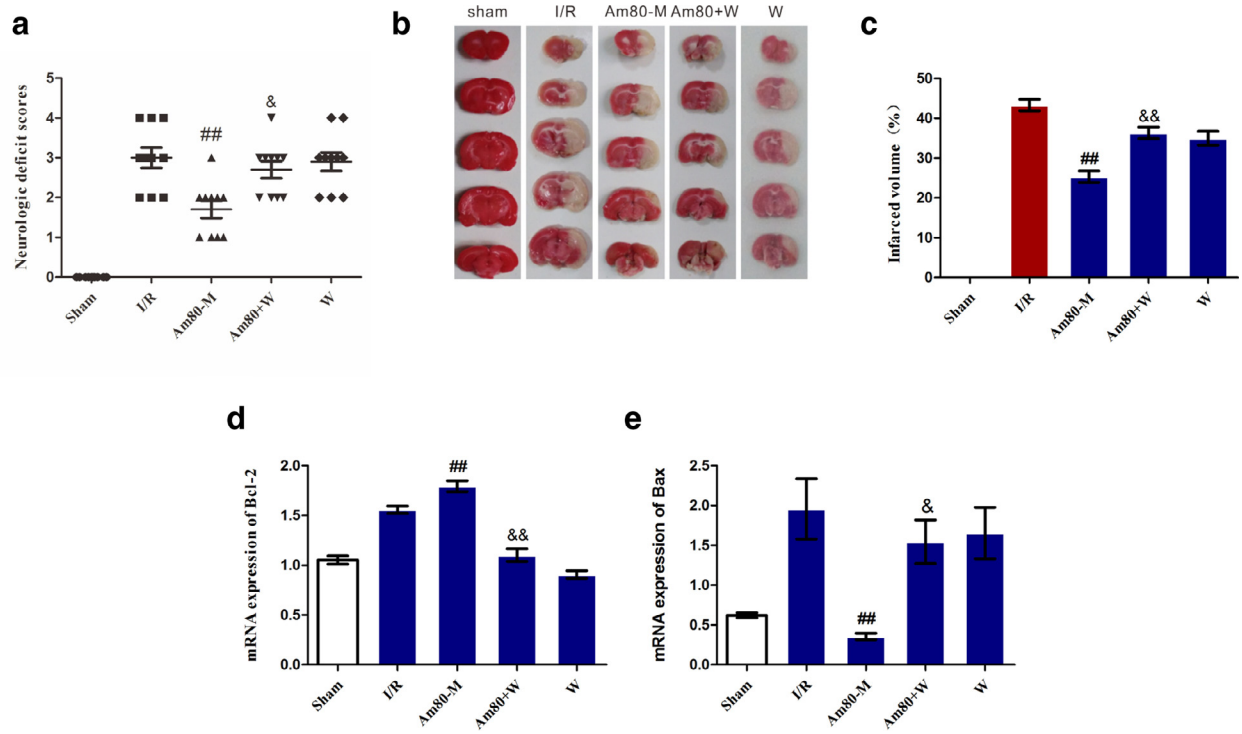

Figure 6. ***Wortmannin inhibits the neuroprotective effects of Am80 treatment.*** *(a) Wortmannin reverses the reduction of neurologic deficit scores induced by Am80 treatment (n = 10). (b) TTC pictures of rats in each group, (c) Infarct volume in rats in each group (n = 10). (d) E expression of Bcl-2 mRNA, (e) Expression of Bcl-2 mRNA at 24 hours after MCAO (n = 6). Values are expressed as the mean ± SEM. ANOVA $^{\#\#}P < .01$ versus I/R, $^{\&}P < .05$, $^{\&\&}P < .01$ versus Am80-M. Abbreviation: ANOVA, analysis of variance; MCAO, middle cerebral artery occlusion; TTC, triphenyltetrazolium chloride.*

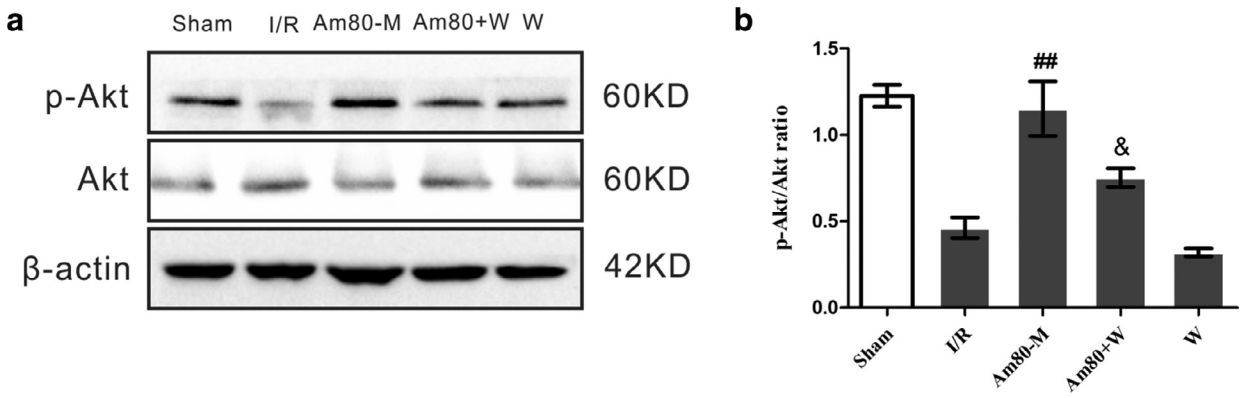

Figure 7. ***Wortmannin inhibits the phosphorylation of Akt after ischemia induced by 24 hours of reperfusion.*** *(a) Representative bands show inhibition of the phosphorylation of Akt by wortmannin (n = 6). (b) Relative density of p-Akt to total-Akt. Values are expressed as the mean ± SEM. ANOVA* $^{\#\#}P < .01$ *versus I/R,* $^{\&}P < .05$ *versus Am80-M. Abbreviation: ANOVA, analysis of variance; I/R, ischemia-reperfusion.*

Numerous studies have demonstrated that neuronal apoptosis has a critical role in the development of brain damage induced by ischemia.[3] TUNEL staining showed that Am80 pretreatment significantly decreased the number of apoptotic neurons after MCAO and reperfusion. In search of the mechanism implicated in this protective effect, the expression of pro- and antiapoptotic proteins was examined. Bcl-2 and Bax represent crucial molecules controlling neuronal apoptosis after cerebral ischemia.[26] Bcl-2, which helps maintain the integrity of membrane, is one of the first identified antiapoptotic factors and is a principal neuroprotective protein in the central nervous system. Bax, also known as a Bcl-2 related protein X, promotes cell apoptosis.[27] In a normal physiological state, Bcl-2 and Bax remain in a balance. However, when cells are exposed to various pathologic stimuli, such as ischemia or anoxia, the expression of Bcl-2 and Bax changes and a decrease in the Bcl-2-to-Bax ratio favors cell apoptosis. Thus, apoptosis is inhibited when Bcl-2 expression dominates and promoted when Bax expression prevails.[28,29] Based on this premise, we have examined the ability of Am80 to upregulate Bcl-2 and downregulate Bax after I/R injury. As expected, the pretreatment with Am80 evidently decreased the expression of Bax and increased the expression of Bcl-2, suggesting that Am80 may inhibit apoptosis in the ischemic hippocampus.

The PI3K/Akt pathway is a critical component of a wide range of mechanisms implicated in the control of cell survival,[30] including ischemic events. It has been documented that in focal cerebral ischemia activated Akt exerts antiapoptotic effects by phosphorylation of its substrates, such as Bcl-2-associated death protein, forkhead transcription factor, and glycogen synthase kinase 3β.[31,32] On this basis, the present work evaluated the phosphorylation of Akt, measured as the ratio of Akt phosphorylated at serine 473 to the total Akt protein. This analysis demonstrated that Am80 at concentrations of 6 and 10 mg/kg markedly enhanced the phosphorylation of Akt in the ischemic hippocampus, producing a neuroprotective effect (Fig 5). Of note, activation of Akt can be achieved in a PI3K-dependent or PI3K-independent manner.[33] To determine whether activation of Akt by Am80 is PI3K-dependent or not, an irreversible PI3K inhibitor, wortmannin, was administered. This experiment demonstrated that wortmannin increased the neurologic deficit and infarct volume, downregulated antiapoptotic Bcl-2, upregulated proapoptotic Bax, and inhibited the phosphorylation of Akt, essentially reversing the beneficial effects of Am80 pretreatment. Thus, the phosphorylation of Akt induced by Am80 is PI3K-dependent, and PI3K/Akt signaling pathway is essential for the antiapoptotic effect of Am80 in cerebral ischemic injury. These findings are in agreement with the well-documented notion that the PI3K/Akt signaling has antiapoptotic effects and promotes neuronal survival after ischemic damage.

Several studies have documented that RARα regulates the PI3K/Akt signaling pathway in various types of cells. For example, Am80 significantly upregulates RARα expression, inducing KLF5 dephosphorylation and inhibiting the interaction of KLF5 with RARα via the PI3K/Akt pathway in vascular smooth muscle cells.[34] Activation of RAR results in phosphorylation of Akt phosphorylation and induction of expression of PI3K catalytic subunit p110β protein, increasing PI3K activity and sequential phosphorylation of Akt and eNOS.[35] Similarly, ligand binding to RAR facilitates the association of p110, the catalytic subunit of PI3K, to that complex, promoting PI3K activity.[36] Finally, the regulation of migration of smooth muscle cells in airways by RAR involves the PI3K/Akt and not the ERK pathway.[37]

In summary, this study demonstrated that the pretreatment with Am80 protects the hippocampus against ischemic injury by modifying the expression of apoptosis-related proteins and this effect may be due, at least in part, to the activation of the PI3K/Akt signaling pathway. However, some limitations of the study should be acknowledged. The effect of Am80 pretreatment was examined only at 24 hours

after reperfusion and whether this pretreatment affords protection at later time points remains unknown. Additionally, further research is necessary to elucidate the relation between the Am80-induced activation of RARα and downstream activation of PI3K/Akt. This effort may provide novel insights regarding the application of Am80 as a neuroprotective agent.

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